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14. ABSTRACT

Breast cancer is the most common cancer diagnosed among women. Breast cancer cells often require activation of the estrogen-specific steroid hormone receptor by estrogen to proliferate, and it is well known that steroid hormone receptor signaling plays a pivotal role in progression of breast cancer disease. We hypothesize that a completely novel conceptual approach known as Protac (Proteolysis Targeting Chimeric Molecule) will lead to the ubiquitination and degradation of targeted proteins. Protacs contain at one end, the IκBα phosphopeptide that binds to a ubiquitin ligase $SCF^{\beta\text{-}TRCP}$ and at the other end, the ligand estradiol. We previously demonstrated that Protacs promote the ubiquitination and degradation of ER and AR *in vitro* and *in vivo*, respectively. While we have demonstrated that a phosphopeptide containing Protac is effective, this compound is not likely to be cell membrane-permeable. The overall goal is to test the hypothesis that small non-peptidic ligands that bind $SCF^{\beta TRCP}$ can be used to generate estrogen-based Protacs that mediate ER ubiquitination and degradation. In Aim 1, we will express recombinant β-TRCP. In Aim 2, we will screen a chemical library for molecules that bind β-TRCP. Potential "hits" we will used to develop new Protacs that will be tested for effects on ER ubiquitination and degradation in breast cancer cells. Protacs provide a novel approach to the treatment of breast cancer.

15. SUBJECT TERMS
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Introduction

Breast cancer is the most common cancer diagnosed among women. Breast cancer cells often require activation of the estrogen-specific steroid hormone receptor by estrogen to proliferate, and it is well known that steroid hormone receptor signaling plays a pivotal role in progression of breast cancer disease. In this proposal, we hypothesized that a completely novel conceptual approach known as Protac (Proteolysis Targeting Chimeric Molecule) will lead to the ubiquitination and degradation of targeted proteins. At one end, Protac contains the IκBα phosphopeptide that binds the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$; at the other end, Protac contains the ligand for ER, estradiol. We previously demonstrated that Protacs promote the ubiquitination and degradation of ER and AR *in vitro* and *in vivo*, respectively.

Body

We proposed to develop a bioassay screen for novel b-TRCP ligands based on the displacement of β-TRCP-bound diphosphorylated peptide. The readout for the proposed screening assay will be *fluorescence polarization* (FP). FP is based on the concept of molecular movement and rotation. By using a fluorescent dye to label the diphosphorylated peptide, its binding to β-TRCP can be monitored through its speed of rotation. If the test compound binds to the same site on β-TRCP, as does the fluorescently labeled peptide, then the liberated fluorescent peptide will rotate or tumble faster, and the resulting emitted light is depolarized relative to the excitation plane. Therefore, the degree of fluorescence polarization in the presence of test compound indicates the level of displacement of the fluorescently labeled peptide, and thus the relative strength of binding to β-TRCP. We had two aims. For Aim 1, a cDNA encoding β-TRCP will be subcloned into different expression vectors to identify a suitable strategy for expression of recombinant protein. We will test expression of β-TRCP in common expression systems including *E. coli*, *Pichia Pastoris*, and insect Sf9 or Hi5 cells. For Aim 2, an18 amino acid diphosphorylated peptide from IκBα will be synthesized, HPLC-purified, and coupled via its amino terminus to the activated ester of the Molecular Probes fluorescent dye. A small molecule library of 10,000 compounds (TimTec, Inc.) is arrayed in a 384 well format in DMSO. Each compound (10μM final concentration) will be tested for the ability to displace the fluorescently labeled peptide from recombinant β-TRCP as measured in a Wallac Victor^2 V fluorescence polarization plate reader. Confirmed positive 'hits' will be scrutinized for 1) ease of chemical synthesis, and 2) ease of derivatization to form a Protac that contains estradiol. These new Protacs will then be tested for their effects on ER degradation and growth of breast cancer cells.

Since the grant was funded, we have been generating the purified β-TRCP. Due to technical difficulties this took longer than expected. We have synthesized the IkBa peptide and are awaiting the coupling to the fluorescent dye. We will be ready to being the screening as described in Aim 2 in the near future.

Key Research Accomplishments
Papers:

Schneekloth JS, Fonseco F, Koldobskiy M, Mandal A, Deshaies RJ, Sakamoto KM, CM Crews. Chemical Genetic Control of Protein Levels: Selective in vivo Targeted Degradation. J Amer Chem Soc, 126(12); 3748-3754, 2004.

Verma R, Peters NR, Tochtrop G, Sakamoto KM, D'Onofrio, Varada R, Fushman D, Deshaies RJ, and RW King. Ubistatins, a Novel Class of Small Molecules that inhibit Ubiquitin-Dependent Proteolysis by Binding to the Ubiquitin Chain. Science, 306:117-120, 2004.

Sakamoto KM. Chimeric Molecules to Target Proteins for Ubiquitination and Degradation. Methods in Enzymology (Ubiquitin and Proteasome System), *in press*.

Reportable Outcomes

1. 3 manuscripts (see above and appendix)
2. Received a Ph.D. from Caltech in 2004.
3. National Institutes of Health (R21 CA108545), Ubiquitination and Degradation in Cancer Therapy 7/1/04-6/31/06 (K. Sakamoto, P.I.)
4. DOD Postdoctoral Fellowship and Fulbright postdoctoral fellowship, Agustin Rodriguez.

Conclusions

Although our screening is in the preliminary stages, our results suggest that the purification of β-TRCP is possible and provide a useful reagent to screen chemical libraries.

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N/A

Appendices

1. Schneekloth JS, Fonseco F, Koldobskiy M, Mandal A, Deshaies RJ, Sakamoto KM, CM Crews. Chemical Genetic Control of Protein Levels: Selective in vivo Targeted Degradation. J Amer Chem Soc, 126(12); 3748-3754, 2004.

2. Verma R, Peters NR, Tochtrop G, Sakamoto KM, D'Onofrio, Varada R, Fushman D, Deshaies RJ, and RW King. Ubistatins, a Novel Class of Small Molecules that inhibit Ubiquitin-Dependent Proteolysis by Binding to the Ubiquitin Chain. Science, 306:117-120, 2004.

3. Sakamoto KM. Chimeric Molecules to Target Proteins for Ubiquitination and Degradation. Methods in Enzymology (Ubiquitin and Proteasome System), *in press*.

4. Curriculum Vitae

Supporting data
Not applicable.

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Chemical Genetic Control of Protein Levels: Selective in Vivo Targeted Degradation

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Abstract: Genetic loss of function analysis is a powerful method for the study of protein function. However, some cell biological questions are difficult to address using traditional genetic strategies often due to the lack of appropriate genetic model systems. Here, we present a general strategy for the design and syntheses of molecules capable of inducing the degradation of selected proteins in vivo via the ubiquitin−proteasome pathway. Western blot and fluorometric analyses indicated the loss of two different targets: green fluorescent protein (GFP) fused with FK506 binding protein (FKBP12) and GFP fused with the androgen receptor (AR), after treatment with PROteolysis TArgeting Chimeric moleculeS (PROTACS) incorporating a FKBP12 ligand and dihydrotestosterone, respectively. These are the first in vivo examples of direct small molecule-induced recruitment of target proteins to the proteasome for degradation upon addition to cultured cells. Moreover, PROTAC-mediated protein degradation offers a general strategy to create "chemical knockouts," thus opening new possibilities for the control of protein function.

Introduction

The selective loss of critical cellular proteins and subsequent analysis of the resulting phenotypes have proven to be extremely useful in genetic studies of in vivo protein function. In recent years, genetically modified knockout cell lines and animals have allowed biological research to advance with unprecedented speed. Chemical genetic approaches, using small molecules to induce changes in cell phenotype, are complementary to traditional genetics. Many chemical genetic strategies use knowledge gained from natural product mode of action studies,[1−3] while others employ chemical inducers of dimerization to manipulate intracellular processes.[4−7] To date, however, there have been few attempts to design small molecules which induce the destruction (rather than inhibition) of a targeted protein in an otherwise healthy cell. Access to such reagents would provide a chemical genetic alternative to the traditional ways of interfering with protein function, resulting in "chemical knockouts". Importantly, a small molecule capable of inducing this process could do so without any genetic manipulation of the organism, thus allowing one to target proteins that are not readily accessible by traditional genetic means (i.e., genes essential for proliferation and early development).

Protein expression can be described as occurring on three levels: DNA, RNA, and post-translation. Consequently, interference with protein function may be approached from each of these levels. Genetic knockouts disrupt protein function at the DNA level by directly inactivating the gene responsible for a protein product. On the RNA level, removal of a protein of interest may be accomplished by RNA interference (RNAi). RNAi causes the degradation of mRNA within the cell, preventing the synthesis of a protein, and often resulting in a "knockdown" or total knockout of protein levels. Interference with gene products at the post-translational level would involve degradation of the protein after it has been completely expressed. To date, interference with proteins on the post-translation level is the least explored.

In principle, targeted proteolytic degradation could be an effective way to accomplish the removal of a desired gene product at the post-translational level. Given the central role of

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the ubiquitin−proteasome pathway in protein degradation within the cell,[8] reagents capable of redirecting the substrate specificity of this pathway would be useful as experimental tools for modulating cellular phenotype and potentially act as drugs for inducing the elimination of disease-promoting proteins. We present here a general strategy for designing molecules capable of inducing the proteolysis of a targeted protein via the ubiquitin−proteasome pathway, as well as the first evidence that such molecules are effective upon addition to living cells.

Protein degradation, like protein synthesis, is an essential part of normal cellular homeostasis. As the major protein degradation pathway, the ATP-dependent ubiquitin−proteasome pathway has been implicated in the regulation of cellular processes as diverse as cell cycle progression,[9] antigen presentation,[10] the inflammatory response,[11] transcription,[12] and signal transduction.[13] The pathway involves two discrete steps: (i) the specific tagging of the protein to be degraded with a polyubiquitin chain and (ii) the subsequent degradation of the tagged substrate by the 26S proteasome, a multicatalytic protease complex. Ubiquitin, a highly conserved 76 amino acid protein,[14] is conjugated to the target protein by a three-part process. First, the C-terminal carboxyl group of ubiquitin is activated by a ubiquitin-activating enzyme (E1). The thioester formed by attachment of ubiquitin to the E1 enzyme is then transferred via a transacylation reaction to an ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is transferred to a lysine (or, less commonly, the amino terminus) of the protein substrate that is specifically bound by an ubiquitin ligase (E3).[15] Successive conjugation of ubiquitin to internal lysines of previously added ubiquitin molecules leads to the formation of polyubiquitin chains.[16] The resulting polyubiquitinated target protein is then recognized by the 26S proteasome, whereupon ubiquitin is cleaved off and the substrate protein threaded into the proteolytic chamber of the proteasome. Importantly, substrate specificity of the ubiquitin−proteasome pathway is conferred by the E3 ligases. Each E3 ligase or recognition subunit of a multiprotein E3 ligase complex binds specifically to a limited number of protein targets sharing a particular destruction sequence. The destruction sequence may require chemical or conformational modification (e.g., phosphorylation) for recognition by E3 enzymes.[17,18]

Recently, we demonstrated a strategy for inducing the ubiquitination and ensuing proteolytic degradation of a targeted protein in vitro. This approach uses heterobifunctional molecules known as PROteolysis TArgeting Chimeric moleculeS (PROTACS), which comprise a ligand for the target protein, a linker moiety, and a ligand for an E3 ubiquitin ligase.[19] In that proof of principle experiment the degradation of a stable protein, methionine aminopeptidase 2 (MetAP-2), was induced in a cellular lysate upon the addition of a PROTAC (referred to as PROTAC-1) consisting of the known MetAP-2 ligand, ovalicin, joined to a peptide ligand for the ubiquitin ligase complex $SCF^{\beta TrCP}$. By bridging MetAP-2 and an E3 ligase, PROTAC-1 initiated the ubiquitination and proteasome-mediated degradation of MetAP-2 (Figure 1). We have also recently shown that an estradiol-based PROTAC (PROTAC-2) could promote the ubiquitination of the human estrogen receptor (hERα) in vitro. Furthermore, a dihydrotestosterone (DHT)-based PROTAC (PROTAC-3), when microinjected into cells, was capable of inducing the degradation of the androgen receptor.[20] Encouraged by our success with PROTACS-1, −2, and −3, we next directed our efforts toward the design of molecules capable of inducing proteolysis simply upon addition to cells. Additionally, the design of new PROTACS takes into account the desire to minimize the amount of molecular biological manipulations necessary to effect degradation to perturb the system as little as possible outside the desired degradation.

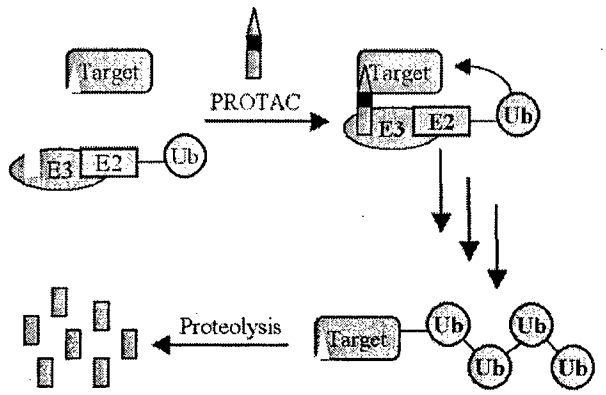

Figure 1. Targeted proteolysis using a PROTAC molecule. Ub = ubiquitin, target = target protein, E3 = E3 ubiquitin ligase complex, and E2 = E2 ubiquitin transfer enzyme.

Results

Development of a Cell Permeable PROTAC: PROTAC-4. For the design of PROTAC-4, we used a protein target/ligand pair developed by ARIAD Pharmaceuticals. The F36V mutation of FK506 binding protein (FKBP12) generates a "hole" into which the artificial ligand AP21998 (**1**) fits via a hydrophobic "bump," thus conferring specificity of this particular ligand to the mutant FKBP over the wild-type protein.[21,22] Inclusion of AP21998 as one domain of PROTAC-4 thus allows it to target (F36V)FKBP12 proteins orthogonally, without disrupting endogenous FKBP12 function. Given the lack of small-molecule E3 ubiquitin ligase ligands, the seven amino acid sequence ALAPYIP was chosen for the E3 recognition domain. This sequence has been shown to be the minimum recognition domain for the von Hippel−Lindau tumor suppressor protein (VHL),[23] part of the VBC−Cul2 E3 ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of hypoxia inducible factor 1α (HIF1α) at P564[24]

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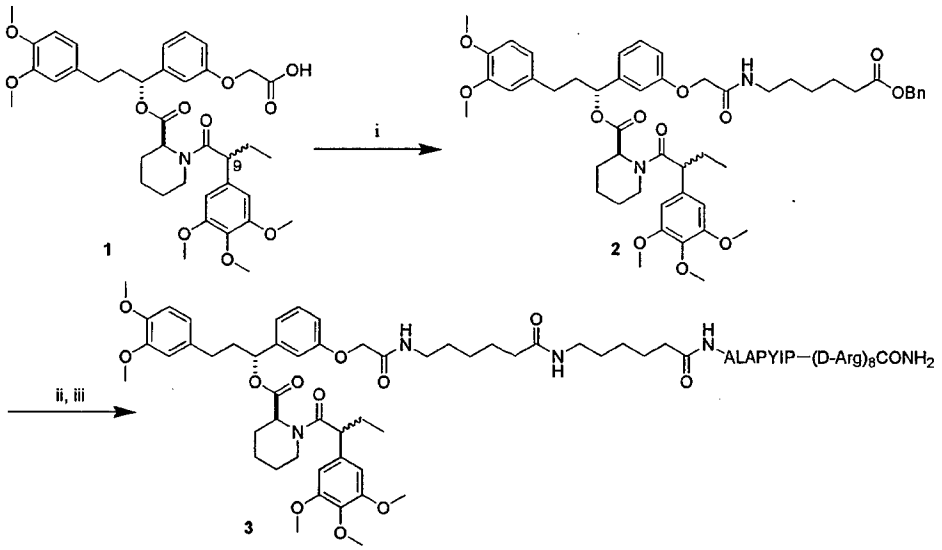
Scheme 1. Synthesis of the AP21998/HIF1 α-Based PROTAC[a]



[a] (i) $H_2N(CH_2)_5CO_2Bn$, EDCI, DMAP. (ii) H_2, Pd/C. (iii) $H_2N(CH_2)_5CONH$-ALAPYIP-(D-Arg)$_8$-NH_2, PyBrOP, DIPEA, DMF.

(the central proline in the ALAPYIP sequence), resulting in recognition and polyubiquitination by VHL. HIF1α is thus constitutively ubiquitinated and degraded under normoxic conditions.[25,26] Finally, a poly-D-arginine tag was included on the carboxy terminus of the peptide sequence to confer cell permeability and resist nonspecific proteolysis. Polyarginine sequences fused to proteins have been shown to facilitate translocation into cells[27,28] via a mechanism that mimics that of the Antennapedia[29] and HIV Tat proteins.[30] Because a molecule fused to the polyarginine sequence should in principle be cell permeable, the necessity of PROTAC microinjection is circumvented. This design element also allows greater flexibility in the types of ligands that could be used in future PROTACs, since polarity of the compound is no longer an issue for membrane permeability. It was hypothesized that PROTAC-4 would enter the cell, be recognized and hydroxylated by a prolyl hydroxylase, and subsequently be bound by both the VHL E3 ligase and the mutant FKBP12 target protein. PROTAC-mediated recruitment of FKBP12 to the VBC−Cul2 E3 ligase complex would be predicted to induce FKBP12 ubiquitination and degradation as in Figure 1.

The F36V FKBP12 ligand AP21998 (**1**) was synthesized as previously described,[21,22] as an approximately 1:1 mixture of diastereomers at C9. Treatment of **1** with the benzyl ester of aminocaproic acid followed by removal of the benzyl group afforded **2** in 85% crude yield after two steps. It is important to note that although this material was carried through as a mixture of two diastereomers at C9, each diastereomer has previously been shown to bind to the target.[22] Standard peptide coupling conditions were used to label the peptide sequence. HPLC purification yielded **3** (PROTAC-4) with 17% recovery from **1** (Scheme 1).

To monitor the abundance of the targeted protein, a vector capable of expressing the mutant FKBP12 fused to enhanced green fluorescent protein (EGFP) was generated. In this way, proteolysis of FKBP12 could be monitored by loss of intracellular fluorescence. This vector was then used to generate a HeLa cell line stably expressing the EGFP−(F36V)FKBP12. Bright field and fluorescent photographs of the cells were taken before and 2.5 h after treatment with PROTAC-4 (**3**). As shown in Figure 2A−D, EGFP−FKBP12 was retained in those cells treated with DMSO, but lost in cells treated with 25 μM PROTAC-4 for 2.5 h. Western blot analysis of cells treated with PROTAC-4 also indicated loss of EGFP−FKBP12 relative to an equal number of cells treated with DMSO (Figure 2I). As a control, cells were treated with uncoupled **1** and the HIF-polyarginine peptide fragment (Figure 2E,F). These cells retained fluorescence, indicating that the two domains require a chemical bond to each other to exert a biological effect. To investigate whether VHL was required for PROTAC-4-mediated EGFP−FKBP12 degradation, the renal carcinoma cell line 786-O[31] was used. 786-O cells failed to produce VHL protein and thus lack a functional VBC−Cul2 E3 ligase complex. 786-O cells stably expressing the degradation substrate EGFP−FKBP12 retained fluorescence despite treatment with 25 μM PROTAC-4 for 2.5 h (Figure 2G,H), confirming that the E3 ligase is required for PROTAC-4 activity. Finally, similar cell density and morphology in bright field images before (Figure 2I) and after (Figure 2J) treatment with 25 μM PROTAC-4 for

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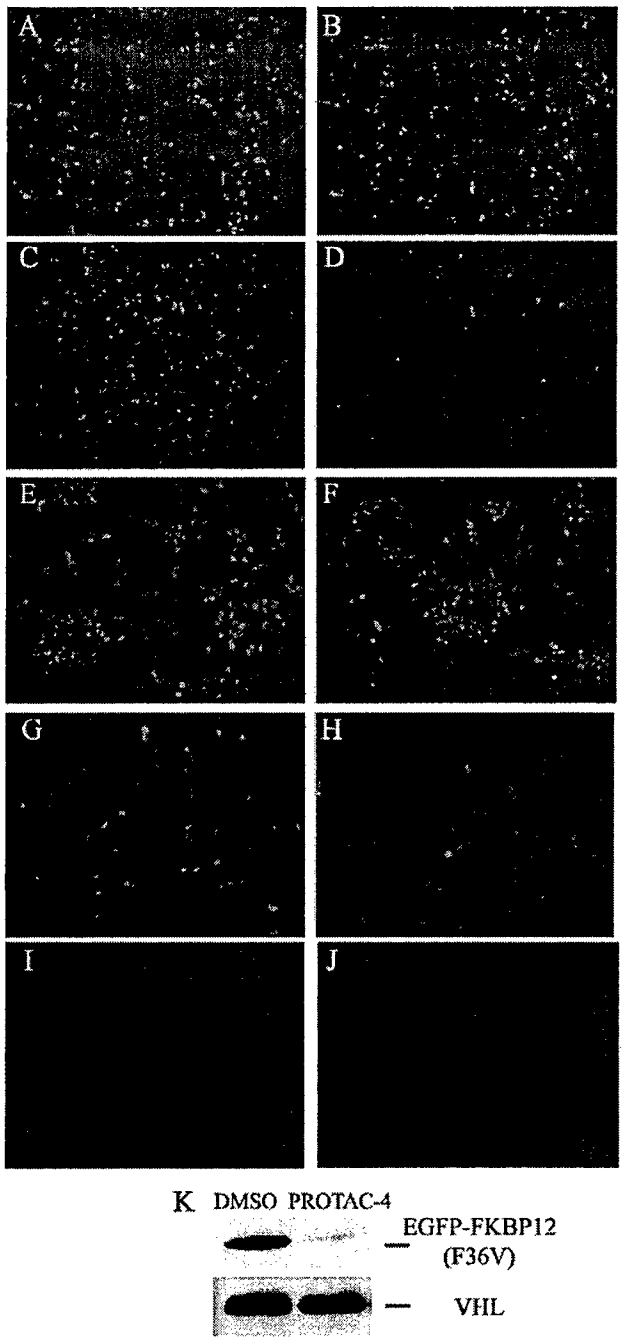


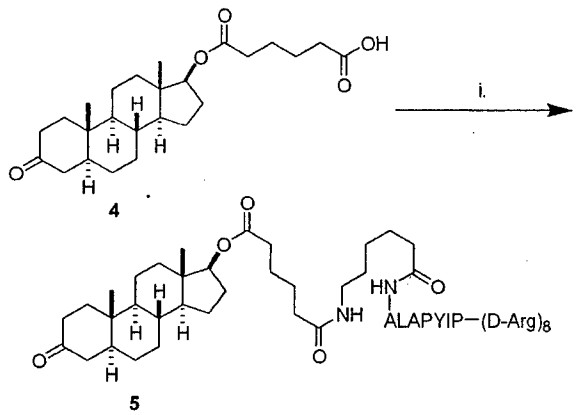
Figure 2. PROTAC-4 (**3**) mediates EGFP–FKBP degradation in a VHL-dependent manner. No change in fluorescence is observed before (A) and 2.5 h after (B) treatment in DMSO control, while a significant change is observed between before (C) and 2.5 h after (D) treatment with 25 μM **3**. Cells treated with 25 μM **1** and 25 μM HIF-(D-Arg)$_8$ peptide show no difference before (E) and 2.5 h after (F) treatment. 786-O$^{EGFP-FKBP}$ cells do not lose fluorescence before (G) or 2.5 h after (H) treatment with 25 μM **3**. Bright field images of cells before (I) and 2.5 h after (J) treatment with 25 μM **1** affirm constant cell density and morphology. Western blot analysis (K) with monoclonal anti-GFP antibodies confirms loss of EGFP–FKBP in cells treated with 25 μM **3** (PROTAC-4) for 2.5 h compared to an equal load from vehicle (DMSO) treated cells.

2.5 h confirm that cells are capable of surviving treatment with a PROTAC molecule.

Implementation of a DHT-Based PROTAC: PROTAC-5. To test the robustness of this approach for the induction of intracellular protein degradation, we next used a well understood protein–ligand pair which occurs in nature. The testosterone/androgen receptor pair was particularly attractive because it has been shown that the androgen receptor (AR) can promote the growth of prostate tumor cells, even in some androgen-independent cell lines.[32] In those same cell lines, it has been shown that inhibition of AR represses growth.[32] We hypothesized that a PROTAC could be utilized to degrade AR, potentially yielding a novel strategy to repress tumor growth. With this in mind, the design of PROTAC-5, **5**, contains DHT as the ligand for AR as well as the HIF-polyarginine peptide sequence which was successful with PROTAC-4. Known DHT derivative **4**[33] was successfully coupled to the HIF-polyarginine peptide with standard peptide coupling conditions (Scheme 2). To monitor protein degradation by fluorescence analysis, HEK293 cells stably expressing GFP-AR (293^{GFP-AR}) were treated with increasing concentrations of PROTAC-5. Within 1 h, a significant decrease in GFP-AR signal was observed in cells treated with 100, 50, and 25 μM PROTAC-5, but not in the DMSO control (Figure 3, parts A–F, I, L). Western blot analysis with anti-AR antisera verified the downregulation of GFP-AR in cells treated with 25 μM PROTAC-5 compared to DMSO control or nontreated cells (Figure 3M). PROTAC-5 concentrations lower than 25 μM did not result in GFP-AR degradation (data not shown). Pretreatment of cells with epoxomicin, a specific proteasome inhibitor,[34] prevented degradation of GFP-AR (Figure 3, part H: light field, K: fluorescent), indicating that the observed degradation was proteasome-dependent. This result was also verified by Western blot (Figure 3N). It should be noted that decreased cell density in the epoxomicin experiments are most likely due to the inherent toxicity of epoxomicin itself, rather than from a toxic effect of the PROTAC. This is supported by the viability of cells treated with PROTAC-5, as seen in Figure 3B,C.

Competition experiments with testosterone also inhibited PROTAC-5 from inducing GFP-AR degradation (Figure 4 A-D). In addition, cells treated only with testosterone retained all fluorescence, as did cells treated with the HIF-polyarginine peptide (Figure 4G,H). Finally, cells treated with both testoster-

Scheme 2. Synthesis of a DHT/HIF1 α-Based PROTAC (PROTAC-3)[a]



[a] (i) $H_2N(CH_2)_5CONH$-ALAPYIP-(D-Arg)$_8$-NH_2, EDCI, DMAP, DMF.

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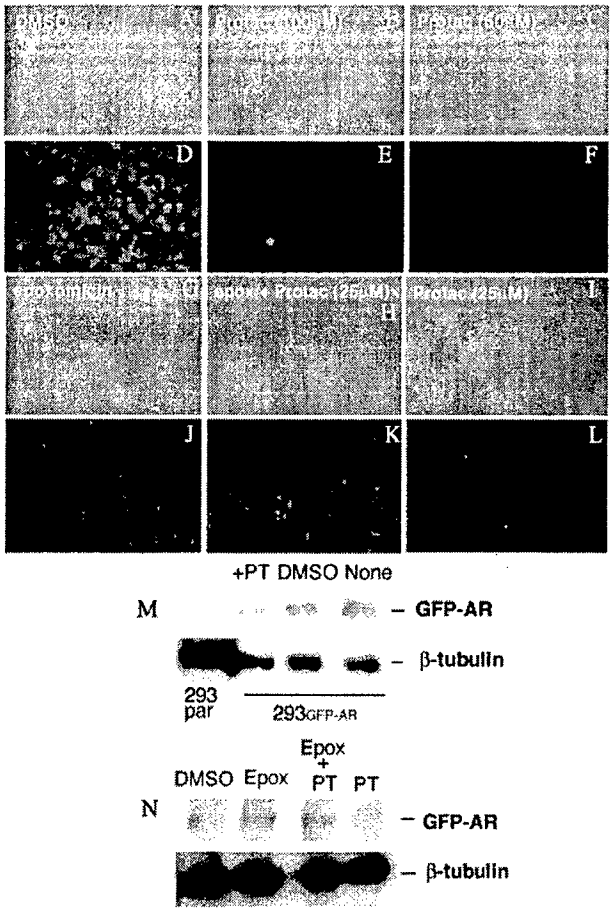

Figure 3. DHT-HIF PROTAC-5 (**5**) mediates GFP-AR degradation in a proteasome-dependent manner. One hour after treatment, 293^{GFP-AR} cells treated with a 100 μM (B light field, E fluorescent) or 50 μM (C light field, F fluorescent) concentration of **5** lose fluorescence, while the DMSO control (A light field, D fluorescent) retains fluorescence. Cells treated with 10 μM epoxomicin (G light field, J fluorescent) and pretreated with 10 μM epoxomicin for 4 h followed by treatment with 25 μM **5** for 1 h (H light field, K fluorescent) retain fluorescence, while cells treated only with 25 μM **5** lose fluorescence after 1 h (I light field, L fluorescent). Western blot analysis confirms loss of GFP-AR after treatment with PROTAC **5** (+PT) relative to a loading control (M), while inhibition of the proteasome with epoxomicin (Epox) inhibits degradation (N).

one and the HIF-polyarginine peptide together also retained fluorescence, indicating again that both domains needed to be chemically linked to observe degradation (Figure 4F). It is important to note again that the cells survived treatment with PROTAC-5, indicating that the strategy of utilizing the ubiquitin–proteasome pathway for targeted degradation does not necessarily cause a toxic effect.

Discussion

These experiments highlight the general applicability of a novel strategy to target and degrade proteins in vivo. Although this technique has been shown to be effective previously in vitro, this is the first example of synthesized molecules which are capable of inducing the degradation of a targeted protein upon addition to cells. Use of a GFP fusion protein provided a convenient method to monitor PROTAC-induced degradation, but is not inherently necessary to the design of the molecule. In principle, no molecular biological manipulations are needed to implement a PROTAC molecule. This technique therefore

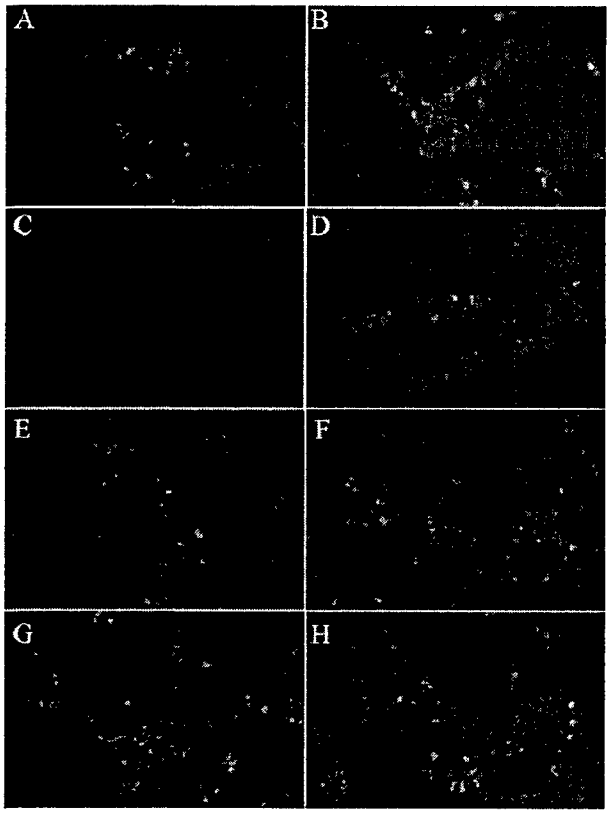

Figure 4. A chemical bond between the HIF-(D-Arg)$_8$ peptide and DHT is required for PROTAC-5-induced degradation of GFP-AR. Cells were treated with (A) no treatment, (B) DMSO (equal volume), (C) 25 μM PROTAC-3, (D) 25 μM PROTAC-5 + 10-fold molar excess testosterone, (E) 25 μM PROTAC-5 + 10-fold molar excess (250 μM) HIF-D-Arg peptide, (F) 25 μM HIF-D-Arg peptide + 25 μM testosterone added separately, (G) 25 μM DHT, and (H) 25 μM HIF-D-Arg peptide.

provides a novel approach to the study of protein function without genetically modifying the host cell. Moreover, the modularity of the PROTAC design offers the possibility to synthesize similar PROTAC molecules targeting a variety of intracellular targets. These experiments have shown that the ligand for the target protein can be varied using both natural and synthetic ligands to degrade effectively targeted GFP fusion proteins. Although the linker length has not been fully explored, a spacer consisting of two aminocaproic acids (12 atoms) has been shown to be flexible enough to accommodate some structural variation in the target and E3 ligase proteins yet remain functional. Since ubiquitination occurs most commonly on an exposed lysine, different spacer lengths may be required to accommodate the structures of different target proteins.

Small molecules have previously been implicated in inducing ubiquitination and degradation of proteins; most notably geldanamycin derivatives act by controlling target interaction with molecular chaperones.[35–38] However, there are often specificity issues with these approaches, and the exact mechanism of

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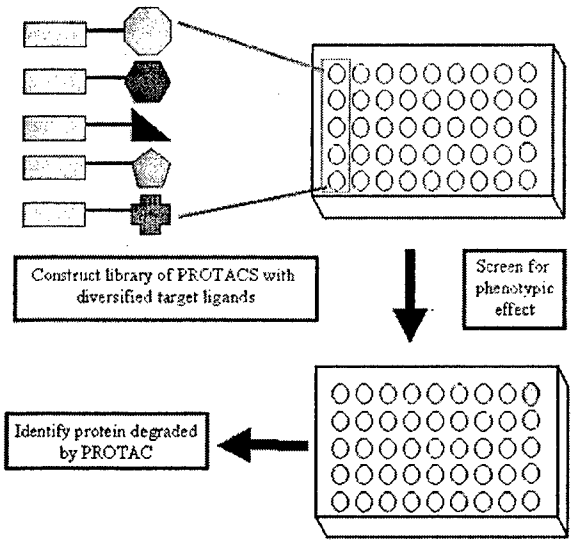

Figure 5. Potential use of PROTACS in a chemical genetic screen.

induced degradation is not clear. Interference with gene products at the post-translational level has also been successfully demonstrated by Howley and co-workers,[39] who used known protein−protein interacting domains. Their approach, while successful, required significant manipulation of the cell lines in question to observe an effect. Both of these methods are significantly less direct and flexible than PROTACS. In addition, the PROTAC strategy represents the first attempt to develop a general method for small molecule-induced targeted proteolysis via the ubiquitin−proteasome pathway in intact cells.

PROTACS could in principle be used to target almost any protein within a cell and selectively initiate its degradation, resulting in a "chemical knockout" of protein function. A notable advantage to this strategy is that proteolysis is not dependent on the active-site inhibition of the target; any unique site of a protein may be targeted, provided that there are exposed lysines within proximity for the attachment of ubiquitin. Because some E3 ligases are expressed in a tissue-specific manner, this also raises the possibility that PROTACS could be used as tissue-specific drugs.

Several other applications for this technology can be envisioned. First, PROTACS could be used to control a desired cellular phenotype, for example, via the induced degradation of a crucial regulatory transcription factor which is difficult to target pharmaceutically. "Chemical knockout" of a protein could prove viable as an alternative for a genetic knockout, which would be extremely valuable in the study of protein function. This strategy could also provide significantly more temporal or dosing control than gene inactivation at the DNA or RNA level. Second, libraries of PROTACS could be used to screen for phenotypic effects in a chemical genetic fashion. This strategy could be used either to identify novel ligands for a target or to identify new therapeutically vulnerable protein targets by studying phenotypic change as a result of selective protein degradation (Figure 5). This chemical genetic strategy would employ a library of PROTAC molecules with identical E3 ubiquitin ligase domains but chemically diverse target ligands. After PROTAC library incubation with cultured cells and detection of the desired cellular phenotype (e.g., inhibition of pro-inflammatory signaling), one could identify the protein that was degraded by incubation with the PROTAC. A number of approaches could be used to identify the PROTAC-targeted protein, including affinity chromatography and differential proteomic technologies such as ICAT.[40] In a modification of this strategy, a library of PROTACS could be screened to identify a ligand for a particular target by monitoring degradation of the target protein (e.g., loss of GFP fusion protein). Finally, PROTACS could be used as drugs to remove toxic or disease-causing proteins. This strategy is particularly appealing since many diseases, including several cancers, are dependent on the presence or overexpression of a small number of proteins. The large number of potential uses for this technology, coupled with the success of these experiments, suggests that PROTACS could find broad use in the fields of cell biology, biochemistry, and potentially medicine.

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Experimental Section

A. Materials. (F36V)FKBP12 expression vector was generously provided by ARIAD Pharmaceuticals (Cambridge, MA), and GFP-AR expression plasmid was a gift from Dr. Charles Sawyers (HHMI, UCLA). Epoxomicin[41] and AP21998[21,22] were synthesized as previously described. Dihydrotestosterone and testosterone were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody recognizing VHL was purchased from Oncogene (San Diego, CA), antibodies recognizing GFP and β-tubulin were obtained from Santa Cruz Biotech (Santa Cruz, CA), and polyclonal antibody against the androgen receptor was from United Biomedical, Inc. (Hauppauge, NY). HEK293, 786-O, and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). Tissue culture medium and reagents were obtained from GIBCO-Invitrogen (Carlsbad, CA).

B. Tissue Culture. HeLa cells, 786-O cells, and HEK 293 cells were separately cultured in D-MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. All cell lines were maintained at a temperature of 37 °C in a humidified atmosphere of 5% CO_2. To generate cells stably expressing a particular fluorescent target protein, the parent cell line was grown to 70% confluency and transfected using calcium phosphate precipitation of the designated cDNA. Following transfection, cells were split 1:10 into culture medium supplemented with 600 μg/mL G418 (GIBCO-Invitrogen). Individual clones which optimally expressed fluorescent target protein were identified and expanded under selection for further experimentation.

C. Detection of PROTAC-Induced Degradation by Fluorescence Microscopy. Cells stably expressing fluorescent target protein were plated into 96 well plates ($HeLa^{EGFP-FKBP}$ cells plated at 4000 cells/well and $HEK293^{GFP-AR}$ cells plated at 60 000−100 000 cells/well). Synthesized PROTACS were dissolved in DMSO vehicle at a final concentration of 1%. Disappearance of target protein in vivo was monitored by fluorescence microscopy at an excitation wavelength of 488 nm.

D. Detection of PROTAC-Induced Degradation by Western Blot. Whole cell lysates were prepared from HeLaEGFP−FKBP cells treated with PROTAC-4 and with HEK293GFP-AR cells treated with PRTOAC-5 by lysing the cells in hot Laemmli buffer. Lysates were subjected to 8% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane. Membranes were blocked in 3% nonfat milk in TBS supplemented with 0.1% Triton X-100 and 0.02% sodium azide. Lysates from $HeLa^{EGFP-FKBP}$ cells treated with

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PROTAC-4 were probed with anti-GFP (1:1000) and anti-VHL (1:1000) antibodies, and HEK293GFP-AR cells treated with PROTAC-5 were probed with anti-androgen receptor (1:1000) and anti-β-tubulin (1:200) antibodies. Blots were developed using chemiluminescent detection.

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Supporting Information Available: Preparation and characterization information for compounds **3** and **5** and the HIF-polyarginine peptide (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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[54] Chimeric Molecules to Target Proteins for Ubiquitination and Degradation

By KATHLEEN M. SAKAMOTO

Abstract

Protein degradation is one of the tactics used by the cell for irreversibly inactivating proteins. In eukaryotes, ATP-dependent protein degradation in the cytoplasm and nucleus is carried out by the 26S proteasome. Most proteins are targeted to the 26S proteasome by covalent attachment of a multiubiquitin chain. A key component of the enzyme cascade that results in attachment of the multiubiquitin chain to the target or labile protein is the ubiquitin ligase that controls the specificity of the ubiquitination reaction. Defects in ubiquitin-dependent proteolysis have been shown to result in a variety of human diseases, including cancer, neurodegenerative diseases, and metabolic disorders.

The SCF (Skp1-Cullin-F-box-Hrt1) complex is a heteromeric ubiquitin ligase that multiubiquitinates proteins important for signal transduction

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and cell cycle progression. A technology was developed known as Protac (*Pro*teolysis *Ta*rgeting Chimeric Molecule) that acts as a bridge, bringing together the SCF ubiquitin ligase with a protein target, resulting in its ubiquitination and degradation. The Protac contains an SCF-binding peptide moiety at one end that is recognized by SCF that is chemically linked to the binding partner or ligand of the target protein. The first demonstration of the efficacy of Protac technology was the successful recruitment, ubiquitination, and degradation of the protein methionine aminopeptidase-2 (MetAP-2) through a covalent interaction between MetAP-2 and Protac. Subsequently, we demonstrated that Protacs could effectively ubiquitinate and degrade cancer-promoting proteins (estrogen and androgen receptors) through noncovalent interactions *in vitro* and in cells. Finally, cell-permeable Protacs can also promote the degradation of proteins in cells. In this chapter, I describe experiments to test the ability of Protacs to target proteins *in vitro* and in cells.

Introduction

Ubiquitin-dependent proteolysis is a major pathway that regulates intracellular protein levels. Posttranslational modification of proteins by E3 ubiquitin ligases results in multiubiquitin chain formation and subsequent degradation by the 26S proteasome (Ciechanover *et al.*, 2000; Deshaies, 1999; Sakamoto, 2002). One potential approach to treating human disease is to recruit a disease-related protein to an E3 ligase for ubiquitination and subsequent degradation. To this end, a technology known as Protacs (*Pro*teolysis *Ta*rgeting Chimeric Molecules) was developed. The goal of Protac therapy is to create a "bridging molecule" that could link together a disease-related protein to an E3 ligase. Protacs consist of one moiety (e.g., a peptide), which is recognized by the E3 ligase. This moiety or peptide is then chemically linked to a binding partner of the target. The idea is that Protacs would bring the target to the E3 ligase in close enough proximity for multiubiquitin attachment, which would then be recognized by the 26S proteasome (Fig. 1). The advantage of this approach is that it is catalytic and theoretically can be used to recruit any protein, even those that exist in a multisubunit complex.

Several applications for Protac therapy are possible. In cancer, the predominant approach to treating patients is chemotherapy and radiation. Both of these forms of therapy result in complications because of effects on normal cells. Therefore, development of therapeutic approaches to specifically target cancer-causing proteins without affecting normal cells is desirable.

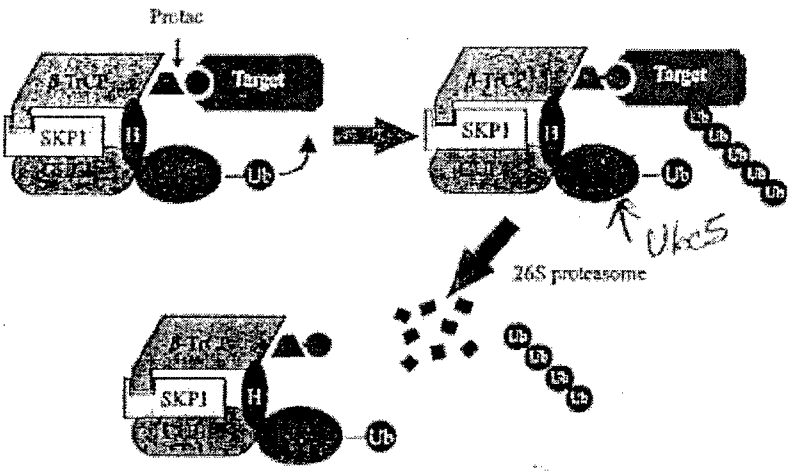


FIG. 1. Protac-1 targets MetAP-2 to SCF. Protac-1 is a chimeric molecule that consists of a phosphopeptide moiety and a small molecule moiety that interacts with the protein target (Sakamoto *et al.*, 2001) (See color insert.)

To test the efficacy of Protacs *in vitro* and *in vivo*, several components are essential. First, a functional E3 ligase is necessary, either in purified form or isolated from cell extracts. Additional components of ubiquitination reaction, including ATP, E1, E2, and ubiquitin, are also required. Second, a small peptide or molecule recognized by the E3 ligase must be identified. Finally, a target with a well-characterized binding partner must be selected that will be chemically linked to the peptide. Finally, successful application of Protacs technology depends on the ability of the Protac to enter cells to target the protein for ubiquitination and degradation. For clinical application, therapeutic drug concentrations are usually considered to be in the nanomolar range.

In addition to the use of Protacs for the treatment of human disease, these molecules provide a chemical genetic approach to "knocking down" proteins to study their function (Schneekloth *et al.*, 2004). The advantages of Protacs are that they are specific and do not require transfections or transduction. Protacs can be directly applied to cells or injected into animals without the use of vectors. Given the increased number of E3 ligases identified by the Human Genome Project, the possibilities for different combinations of Protacs that link specific targets to different ligases are unlimited. This chapter describes general strategies of testing the efficacy of Protacs using two E3 ligases as an example: $SCF^{\beta\text{-}TRCP}$ and Von Hippel

Lindau (VHL) complexes (Ivan *et al.*, 2001; Kaelin, 2002). Three different targets will be described: methionine aminopeptidase-2 (MetAP-2), estrogen receptor (ER), and androgen receptor (AR). We will provide an overview of binding assays, transfections, immunoprecipitations, and ubiquitination and degradation assays of the proteins targeted to ubiquitin ligases by Protacs.

Strategies to Assess the Efficacy of Protacs *In Vitro*

As proof of concept, we generated a Protac molecule that targets the protein MetAP-2 for ubiquitination and degradation. MetAP-2 cleaves the N-terminal methionine from nascent polypeptides and is one of the targets of angiogenesis inhibitors fumagillin and ovalicin (Griffith *et al.*, 1997; [redacted] 1998; Sin *et al.*, 1997). Ovalicin covalently binds to MetAP-2 at the His-231 active site. Inhibition of MetAP-2 is thought to block endothelial cell proliferation by causing G1 arrest (Yeh *et al.*, 2000). MetAP-2 is a stable protein that has not been demonstrated to be ubiquitinated or an endogenous substrate of $SCF^{\beta\text{-TRCP}}$. For these reasons, Met-AP2 was chosen to be the initial target to test Protacs.

The heteromeric ubiquitin ligase, $SCF^{\beta\text{-TRCP}}$ (Skp1-Cullin-Fbox-Hrt1), was selected because the F-box protein β-TRCP/E3RS was previously shown to bind to IκBα (inhibitor of NFκBα) through a minimal phosphopeptide sequence, DRHDS*GLDS*M (phosphoserines indicated by asterisks) (Ben-Neriah, 2002; Karin and Ben-Neriah, 2000). This 10-amino acid phosphopeptide was linked to ovalicin to form the Protac (Protac-1) as previously described (Sakamoto *et al.*, 2001).

MetAP-2-Protac Coupling Assay

MetAP-2 (9 μM) was incubated with increasing concentrations of Protac-1 for 45 min at room temperature (Fig. 2). Reactions were supplemented with SDS loading dye, fractionated on an SDS/10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and immunoblotted with rabbit polyclonal anti-MetAP-2 antisera (Zymed, Inc.). Detection was performed using enhanced chemiluminescence (Amersham, Inc.).

Tissue Culture

293T cells were cultured in DMEM with 10% (vol/vol) FBS (Gibco, Inc.), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM). Cells were split 1:5 before the day of transfection and transiently transfected with 40 μg of plasmid. Cells were 60% confluent in 100-mm

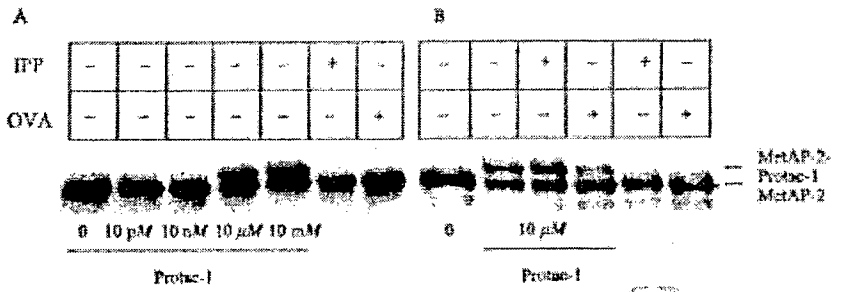


FIG. 2. MetAP-2 binds Protac specifically and in a concentration dependent manner. (A) MetAP-2 (9 μM) was incubated with increasing concentrations of Protac-1 at room temperature for 45 min. The last two lanes depict MetAP-2 that was incubated with either free IκBα phosphopeptide (IPP; 50 μM) or free ovalicin (OVA; 50 μM), as indicated. After incubation, samples were supplemented with SDS-PAGE loading dye, fractionated by SDS-PAGE, and immunoblotted with MetAP-2 antiserum. (B) Same as (A), except MetAP-2 (9 μM) plus Protac-1 (10 μM) were supplemented with either IκBα phosphopeptide (50 μM) or ovalicin (50 μM) as indicated. Protac binding to MetAP-2 was inhibited by addition of ovalicin, but not phosphopeptide (B) (Sakamoto *et al.*, 2001).

dishes on the day of transfection. DNA (20 μg of pFLAG-CUL1 and 20 μg of pFLAG-β-TRCP) was added. Cells were transfected using calcium phosphate precipitation as previously described (Lyapina *et al.*, 1998). Cells were harvested 30 h after transfection. Five micrograms of pGL-1, a plasmid containing the cytomegalovirus (CMV) promoter linked to the green fluorescent protein (GFP) cDNA, was cotransfected into cells to determine transfection efficiency. In all experiments, greater than 80% of the cells were GFP-positive at the time of harvest, indicating high transfection efficiency.

Immunoprecipitations and Ubiquitination Assays

293T cells were lysed with 200 μl of lysis buffer (25 m*M* Tris-Cl, pH 7.5/150 m*M* NaCl/0.1% Triton X-100/5 m*M* NaF/0.05 m*M* EGTA/1 m*M* PMSF). Pellets were lysed by vortexing for 10 sec in a 4° cold room, then placed on ice for 15 min. After centrifugation at 13,000 rpm in a Microfuge for 5 min at 4°, the supernatant was added to 20 μl of FLAG M2 affinity beads (Sigma) and incubated for 2 h rotating at 4°. Beads were spun down at 13,000 rpm and washed with buffer A (25 m*M* Hepes buffer, pH 7.4/0.01% Triton X-100/150 m*M* NaCl) and one wash with buffer B (the same as buffer A but without Triton X-100). Four microliters of MetAP-2 (18 μM) stock, 4 μl of Protac-1 (100 μM), 0.5 μl of 0.1 μg/μl purified mouse E1 (Boston Biochem), 1 μl of 0.5 μg/μl human Cdc34 E2 (Boston Biochem), and 1 μl of

25 m*M* ATP were added to 20 μl (packed volume) of FLAG beads immunoprecipitated with SCF. Reactions were incubated for 1 h at 30° in a Thermomixer (Eppendorf) with constant mixing. SDS-PAGE loading buffer was added to terminate reactions, which were then evaluated by Western blot analysis as previously described (Sakamoto *et al.*, 2001) (Fig. 3). Our results demonstrated that MetAP-2 bound to Protac could be ubiquitinated *in vitro* in the presence of SCF. These methods can be generalized to other ubiquitin ligases provided that a small molecule or peptide ligand exists to enable the synthesis of a suitable Protac and expression vectors that contain tagged versions of the protein or subunits are available. Alternative tags (e.g., myc or HA) have been used, and the resin can be cross-linked with an antibody, which can then be used to immunoprecipitate the E3 ligase from mammalian cells. Both the ER and AR are members of the steroid hormone receptor superfamily whose interactions with ligand (estradiol and testosterone, respectively) have been well characterized (Fig. 4). The ER has been implicated in the progression of breast cancer (Howell *et al.*, 2003). Similarly, hormone-dependent prostate cancer cells grow in response to androgens (Debes *et al.*, 2002). Therefore, both ER and AR are logical targets for cancer therapy. To target ER for ubiquitination and degradation, a Protac

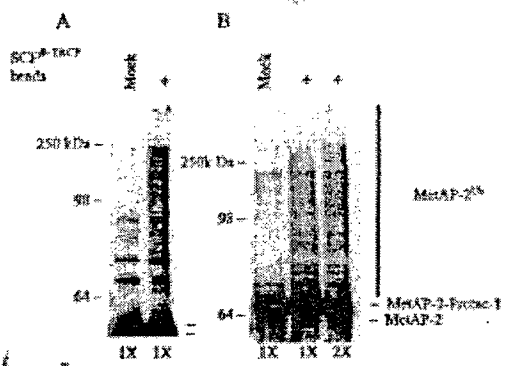


FIG. 3. Protac mediates MetAP-2 ubiquitination by SCF. (A) Ubiquitination of the 46-kDa fragment of MetAP-2. MetAP-2–Protac-1 mixture was added to either control (mock) or SCF$^{\beta\text{-TRCP}}$ beads (+) supplemented with ATP plus purified E1, E2 (Cdc34), and ubiquitin. UbcH5c (500 ng) was also tested as E2 in the reaction, which resulted in the same degree of ubiquitination as observed with Cdc34 (data not shown). Reactions were incubated for 1 h at 30°, and were evaluated by SDS-PAGE followed by Western blotting with anti-MetAP-2 antiserum. (B) Ubiquitination of full-length (67 kDa) MetAP-2. Same as (A), except that the 67-kDa preparation of MetAP-2 was used, and E1, E2, plus ubiquitin were either added at normal (1×) or twofold higher (2×) levels, as indicated (Sakamoto *et al.*, 2001).

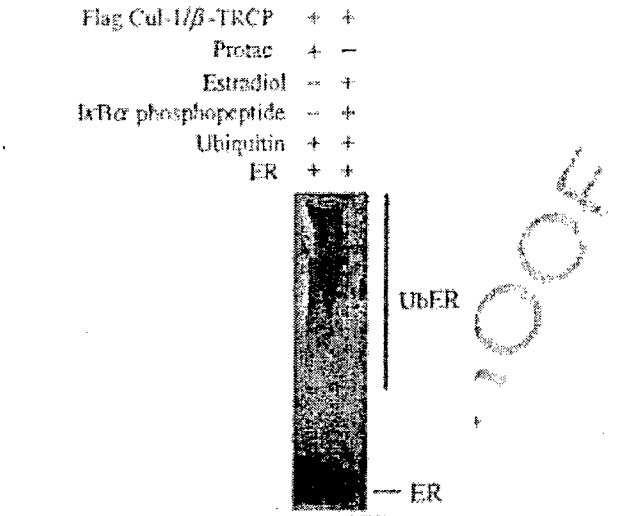


FIG. 4. Protac-2 activates ubiquitination of ER *in vitro*. Purified ER was incubated with recombinant E1, E2, ATP, ubiquitin, and immobilized $SCF^{\beta\text{-}TRCP}$ isolated from animal cells by virtue of Flag tags on cotransfected Cul1 and β-TRCP. Reactions were supplemented with the indicated concentration of Protac-2, incubated for 60 min at 30°, and monitored by SDS-PAGE followed by immunoblotting with an anti-ER antibody (Sakamoto *et al.*, 2003).

(Protac-2) was synthesized, containing the IκBα phosphopeptide linked to estradiol (the ligand for ER) (Sakamoto *et al.*, 2003).

Determination of Protein Degradation of Ubiquitinated Proteins In Vitro

The success of Protacs depends not only on efficient ubiquitination of the proposed target but also degradation of that target in cells. Several approaches can be used both *in vitro* and *in vivo* to demonstrate that the target is being destroyed. First, demonstration of degradation *in vitro* can be performed with purified 26S proteasome. For these experiments, we used purified yeast proteasomes as previously described (Verma *et al.*, 2000, 2002).

Ubiquitination assays were first performed with the immunoprecipitated E3 ligase, purified target, E1, E2, ATP, and ubiquitin with Protac. Purified yeast 26S proteasomes (40 μl of 0.5mg/ml) were added to ubiquitinated protein (e.g., ER) on beads. The reaction was supplemented with 6 μl of 1 m*M* ATP, 2 μl of 0.2 *M* magnesium acetate, and ubiquitin aldehyde (5 μ*M* final concentration). The reaction was incubated for 10 min at 30° with the occasional mixing in the Thermomixer (Eppendorf). To

verify that degradation is due to proteasomes and not other proteases, purified 26S proteasomes were preincubated for 45 min at 30° with 1 mm of 1, 10 phenanthroline (Sigma) (a metal chelator and inhibitor of the RPN11 deubiquitinating enzyme in the 26S proteasome) (Fig. 5).

Strategies to Assess the Efficacy of Protacs *In Vivo*

Clinical application of Protacs is dependent on successful ubiquitination and degradation of the protein target by endogenous ubiquitin ligases and proteasomes within cells. There are several approaches to test the efficacy of Protacs using cell extracts or application directly to cells. Depending on the polarity of the Protac, efficiency of internalization in cells is variable. If Protacs are hydrophilic, such as the case with the Protac-1 that contains the IκBα phosphopeptide, extracts or microinjections are possible approaches. For cell-permeable Protacs, it is be possible to directly bath apply Protacs to cells.

Degradation Experiments with Xenopus *Extracts*

Extracts from unfertilized *Xenopus laevis* eggs were prepared on the day of the experiment as previously described (Murray, 1991). MetAP-2 (4 μl of 9 μ*M*) was incubated with Protac-1 (50 μ*M*) at room temperature for 45

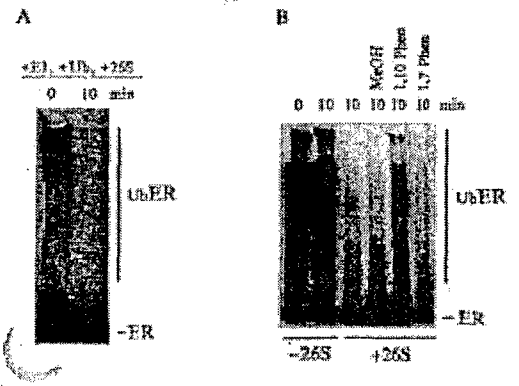


FIG. 5. Ubiquitinated ER is degraded by the 26S proteasome. (A) Ubiquitination reactions performed as described in the legend to Fig. 5A were supplemented with purified yeast 26S proteasomes. Within 10 min, complete degradation of ER was observed. (B) Purified 26S proteasome preparations were preincubated in 1,10 phenanthroline (1 m*M*) or 1,7 phenanthroline (1 m*M*) before addition. The metal chelator 1,10 phenanthroline inhibits the Rpn11-associated deubiquitinating activity that is required for substrate degradation by the proteasome. Degradation of ER was partially inhibited by addition of 1,10 phenanthroline, but not the inactive derivative 1,7 phenanthroline (Sakamoto *et al.*, 2003).

min. The MetAP-2–Protac-1 mixture was added to 10 μl of extract in addition to excess ovalicin (10 μM final concentration). The excess of ovalicin was added to saturate any free MetAP-2 in the reaction. Additional components in the reaction included constitutively active IKK (IKK-EE; 0.4 μg) and okadaic acid (10 μM final concentration) to maintain phosphorylation of the IκBα peptide moiety of Protac. To test for specificity of proteasomal degradation, various proteasome inhibitors were used, including N-acetyl-leu-leu-norleucinal (LLnL, 50 μM final) or epoxomicin (10 μM final). Protease inhibitors chymotrypsin, pepstatin, and leupeptin cocktail (15 μg/ml final concentration) were also added to the extracts. Reactions were incubated for time points up to 30 min at room temperature and terminated by adding 50 μl of SDS loading buffer. Samples were then evaluated by Western blot analysis using MetAP-2 antiserum (Fig. 6).

Microinjection as a Method to Study Effects of Protacs on Ubiquitination and Degradation of Target Proteins

Protacs that contain a phosphopeptide do not enter cells efficiently. Various protein transduction domains, lipid-based transfection reagents, and electroporation or other transient transfection methods can be tested. However, to facilitate Protacs entering cells, microinjections were performed. For these experiments, Protac-3 (IκBα) phosphopeptide-testosterone was synthesized to target the AR (Sakamoto *et al.*, 2003). As a readout of protein degradation, 293 cells stably expressing AR-GFP were selected using G418 (600 μg/ml). Before microinjections, cells were approximately 60% confluent in 6-cm dishes.

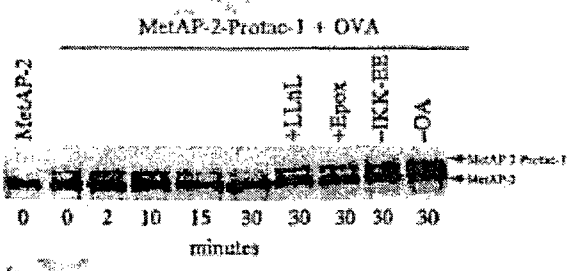
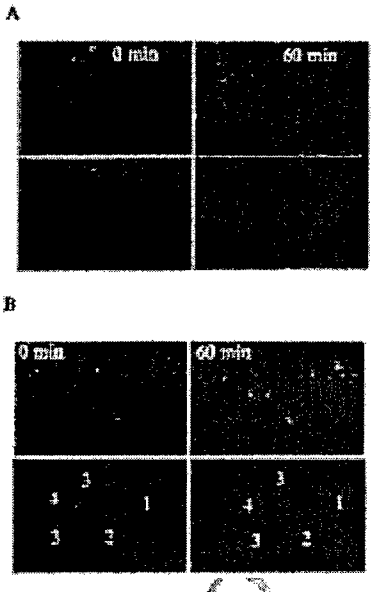


FIG. 6. MetAP-2-Protac but not free MetAP-2 is degraded in *Xenopus* extracts. The MetAP-2–Protac-1 mixture or MetAP-2 alone was added to *Xenopus* egg extract fortified with ovalicin (OVA; 100 μM), IKK-EE (0.4 μg), and okadaic acid (10 μM). Where indicated, reactions were either deprived of IKK-EE or okadaic acid (OA) or were further supplemented with 50 μM LLnL or 10 μM epoxomicin (Epox). Reactions were incubated for the indicated time points at room temperature, terminated by adding SDS-PAGE loading dye, and evaluated by SDS-PAGE followed by Western blotting with anti-MetAP-2 antiserum (Sakamoto *et al.*, 2001).

Protac-3 diluted in a KCl solution (10 μM final) with rhodamine dextran (molecular mass 10,000 Da; 50 μg/ml) was injected into cells through a microcapillary needle using a pressurized injection system (Picospritzer II; General Valve Corporation). Coinjection with rhodamine dextran is critical to ensure that decrease in AR-GFP is not due to leakage of protein from cells after microinjection. The injected volume was 0.2 pl, representing 5–10% of the cell volume. GFP and rhodamine fluorescence can be visualized with a fluorescent microscope (Zeiss) and photographs taken with an attached camera (Nikon). Within 1 h after microinjection, disappearance of AR-GFP is visible (Fig. 7). Cells should remain rhodamine



Degree of AR-GFP Disappearance	Percent (out of >200 cells)
1. NONE	4
2. MINIMAL	16
3. PARTIAL	29
4. COMPLETE	51

FIG. 7. Microinjection of Protac leads to AR-GFP degradation in cells. Protac-3 (10 μM in the microinjection needle) was introduced using a Picospritzer II pressurized microinjector into $293^{AR\text{-}GFP}$ cells in a solution containing KCl (200 μM) and rhodamine dextran (50 μg/ml). Approximately 10% of total cell volume was injected. (A) Protac-3 induces AR-GFP disappearance within 60 min. The top panels show cell morphology under light microscopy overlaid with images of cells injected with Protac as indicated by rhodamine fluorescence (pink color). The bottom panels show images of GFP fluorescence. By 1 h, GFP signal disappeared in almost all microinjected cells. To quantify these results, we injected more than 200 cells and classified the degree of GFP disappearance as being either none (1), minimal (2), partial (3), or complete (4). Examples from each category and the tabulated results are shown in (B). These results were reproducible in three independent experiments performed on separate days with 30–50 cells injected per day (Sakamoto *et al.*, 2003). (See color insert.)

positive provided that injection has not caused lysis of cells or leakage of AR-GFP from cells. Greater than 200 cells per experiment (in three separate experiments) provide data demonstrating that Protacs induces degradation of the target. AR-GFP disappearance can then be quantitated by categorizing the intensity of GRP signal as indicative of complete disappearance, partial disappearance, minimal disappearance, or no disappearance. To verify that the disappearance of AR-GFP from cells is proteasome dependent, cells were pretreated with proteasome inhibitor epoxomicin (10 μM final) for 5 h before microinjections or were coinjected with epoxomicin (10 μM).

Methods to Test a Cell-Permeable Protac

Reagents capable of redirecting the substrate specificity of the ubiquitin-proteasome pathway in protein degradation would be useful experimental tools for modulating cellular phenotype and potentially acting as drugs to eliminate disease-promoting proteins. To use Protacs to remove a gene product at the posttranslational level, a cell-permeable reagent would be necessary. A HIF1α-DHT Protac was developed for this purpose. Given the lack of small molecule E3 ligase ligands, the seven amino acid sequence ALAPYIP from hypoxia-inducible factor 1α (HIF1α) was chosen for the E3 recognition domain of Protac-4 (Schneekloth *et al.*, 2004). This sequence has been demonstrated to be the minimum recognition domain for the von Hippel–Lindau tumor suppressor protein (VHL) (Hon *et al.*,

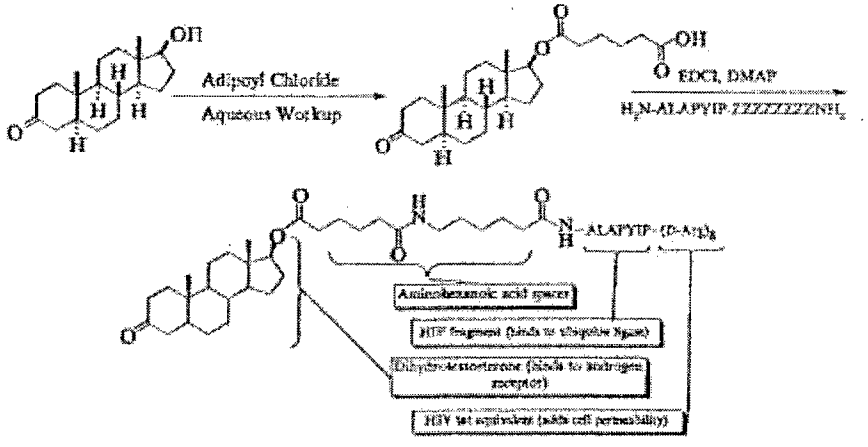


FIG. 8. Chemical Structure of HIF-DHT Protac (Schneekloth *et al.*, 2004).

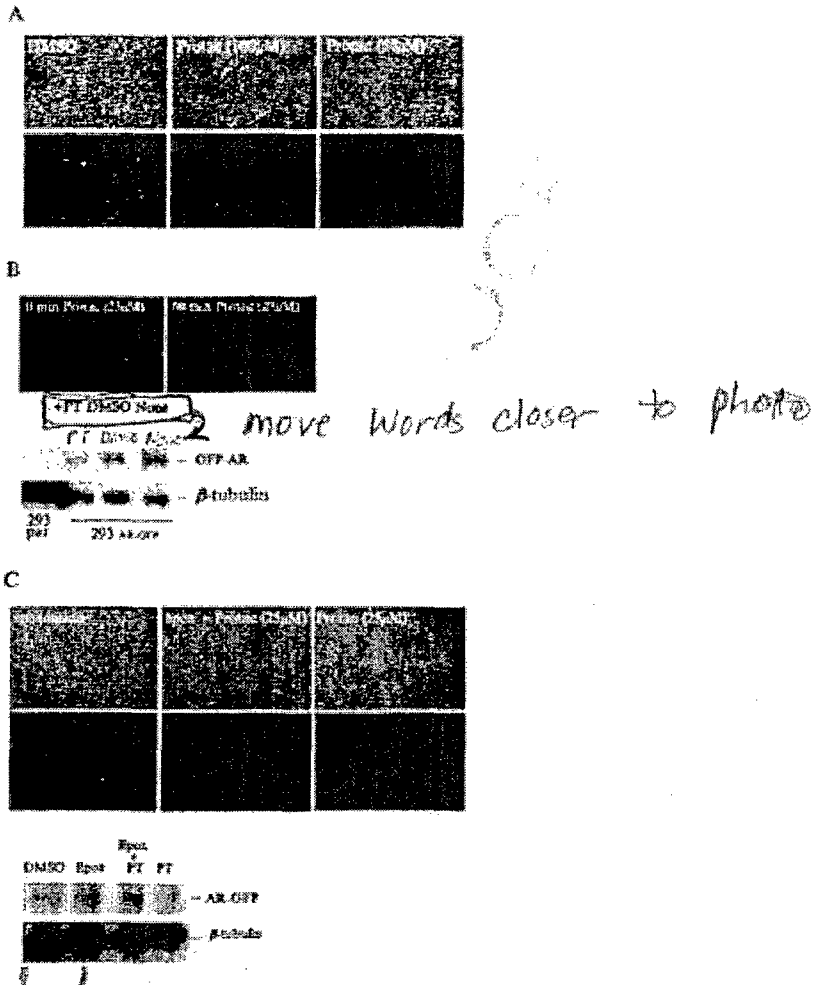


FIG. 9. HIF-DHT Protac mediates AR-GFP degradation in a proteasome-dependent manner; $293^{AR\text{-}GFP}$ cells (0.5×10^6 cells/ml) were plated at 50% confluence in a volume of 200 μl of media in a 96 well dish. (A and B) Protac induces AR-GFP disappearance within 60 min. Protac in a 100-, 50-, or 25-μ*M* concentration or DMSO control in a volume of 0.6 μl was added. Cells were visualized under light (top) or fluorescent (bottom) microscopy 1 h after treatment. Photographs were taken with a SC35 type 12, 35-mm camera attached to an Olympus fluorescent inverted microscope. (B) AR-GFP protein is decreased in cells treated with Protac. Lysates were prepared from parental cells (293 par) or AR-GFP expressing cells treated with Protac (+PT), DMSO, or no treatment (None) for 60 min. Western blot analysis was performed with rabbit polyclonal anti-AR antisera (1:1000; UBI) or β-tubulin (1:200;

2002; Kaelin, 2002). VHL is part of the VBC-Cul2 E3 ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of HIF1α at the (Epstein *et al.*, 2001) central proline in the ALAPYIP sequence. This modification results in recognition and polyubiquitination by VHL. HIF1α is constitutively ubiquitinated and degraded under normoxic conditions (Kaelin, 2002). In addition, a poly-D-arginine tag derived from HIV tat was added to the carboxy terminus of the peptide sequence to confer cell permeability and prevent nonspecific proteolysis (Kirschberg *et al.*, 2003; Wender *et al.*, 2000) (Fig. 8). This Protac should then enter the cell, be recognized and hydroxylated by a prolyl hydroxylase, and subsequently be bound by both the VHL E3 ligase and the target, AR.

The 293 cells stably expressing AR-GFP were used to study the effects of HIF1α-DHT Protac on AR degradation. For these experiments, greater than 95% of cells expressed AR-GFP. On the day before experiments, cells were plated in 96-well plates with 200 μl of media at 60% confluence. Protac was dissolved in DMSO and was added to cells at concentrations ranging between 10 μ*M*–100 μ*M*. The presence or absence of GFP expression after Protac treatment was determined by fluorescent microscopy. A time course was performed, but for HIF1α-DHT Protac, the effects were observed within 2 h. To assess proteasome-dependent degradation, cells were pretreated with epoxomicin (10 μ*M* final concentration) for 4 h before adding Protac. Western blot analysis was performed to determine levels of AR-GFP (Fig. 9).

To measure the protein levels of AR-GFP after Protac treatment, the cells were harvested, washed with PBS once, then pelleted at 1500 rpm. Cells were lysed with boiling SDS loading buffer (30 μl), then boiled for 5 min. Lysates were subjected to 8% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane. Western blot analysis was performed with antiandrogen receptor (1:1000) and anti-beta tubulin (1:200) antisera. Detection was determined using chemiluminescence.

Santa Cruz). (C) Epoxomicin inhibits Protac-induced degradation of AR-GFP. Cells were plated at a density of 0.3×10^6 cells/ml and treated with 10 μ*M* epoxomicin (Calbiochem) or DMSO for 4 h before adding Protac (25 μ*M*) for 60 min. (D) Western blot analysis was performed with cells in 96-well dishes treated with Protac (25 μ*M*), DMSO (left), epoxomicin (10 μ*M*), epoxomicin (10 μ*M*) + Protac (50 or 25 μ*M*), or Protac alone (50 or 25 μ*M*) (Schneekloth *et al.*, 2004). (See color insert.)

Acknowledgments

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Materials and Methods

Figs. S1 to S11
References and Notes

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Ubistatins Inhibit Proteasome-Dependent Degradation by Binding the Ubiquitin Chain

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To identify previously unknown small molecules that inhibit cell cycle machinery, we performed a chemical genetic screen in *Xenopus* extracts. One class of inhibitors, termed ubistatins, blocked cell cycle progression by inhibiting cyclin B proteolysis and inhibited degradation of ubiquitinated Sic1 by purified proteasomes. Ubistatins blocked the binding of ubiquitinated substrates to the proteasome by targeting the ubiquitin-ubiquitin interface of Lys^{48}-linked chains. The same interface is recognized by ubiquitin-chain receptors of the proteasome, indicating that ubistatins act by disrupting a critical protein-protein interaction in the ubiquitin-proteasome system.

Unbiased chemical genetic screens can identify small molecules that target unknown proteins or act through unexpected mechanisms (*1*). To identify previously unknown components or potential drug targets required for cell division, we screened for small molecules that stabilize cyclin B in *Xenopus* cell cycle extracts. Cyclin B degradation regulates exit from mitosis and requires activation of an E3 ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C) (*2*). Because APC/C activation requires mitotic entry, we anticipated that this screen would identify compounds that stabilized cyclin B indirectly by blocking mitotic entry as well as compounds that directly inhibited the cyclin proteolysis machinery. To monitor APC/C activation, we fused the destruction-box domain of *Xenopus* cyclin B1 to luciferase (*3*) and found that the reporter protein was degraded in mitotic but not interphase extracts (fig. S1). Proteolysis was sensitive to inhibitors of cyclin-dependent kinases and the ubiquitin-proteasome system but not affected by inhibitors of DNA replication or spindle assembly, as expected in egg extracts lacking exogenous nuclei (*4*, *5*) (fig. S2).

We developed a miniaturized assay system (*6*) and screened 109,113 compounds to identify 22 inhibitors (Table 1). To distinguish compounds that blocked mitotic entry from direct inhibitors of proteolysis, we arrested extracts in mitosis before addition of the compound and the reporter protein. Sixteen compounds lost inhibitory activity under these conditions (class I, fig. S3), whereas six compounds (class II, fig. S4) remained inhibitory. We next activated proteolysis directly in interphase extracts by adding the APC/C activator Cdh1 (Cdc20 homolog 1) (*7*). Again we found that only class II compounds re-

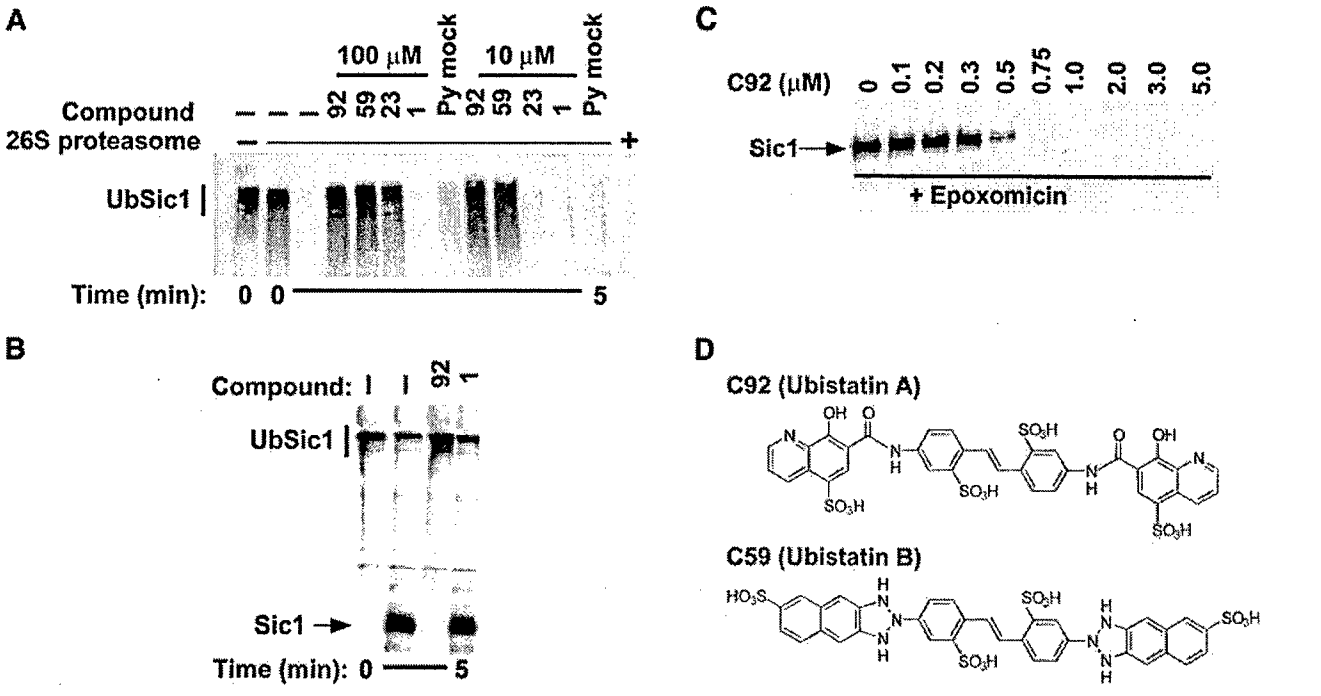

Fig. 1. Class IIB compounds inhibit degradation and deubiquitination of UbSic1 by purified 26S proteasomes. (A) Purified 26S proteasomes were preincubated in the presence or absence of test compounds. UbSic1 was then added and assayed for degradation by immunoblotting for Sic1 (3). Py mock refers to pyridine in which C23 was dissolved. (B) Purified 26S proteasomes were preincubated with 100 μM epoxomicin in the presence or absence of 100 μM test compound. UbSic1 was then added and deubiquitination monitored by immunoblotting for Sic1 (3). (C) Titration of C92 in deubiquitination assay. (D) Structures of C92 and C59 (ubistatins A and B).

tained inhibitory activity. We concluded that class I compounds blocked entry into mitosis or APC/C activation, whereas class II compounds directly blocked components of the cyclin degradation machinery. We next examined whether the inhibitors could block turnover of a β-catenin reporter protein (*8*), a substrate of the SKP1/cullin/F-box protein ($SCF^{\beta\text{-TRCP}}$, where β-TRCP is β-transduction repeat–containing protein) ubiquitin ligase (Table 1). Three class II compounds (class IIB) were inhibitory, suggesting these compounds inhibited a protein required for the degradation of both APC/C and $SCF^{\beta\text{-TRCP}}$ substrates. Class IIB compounds did not block cyclin B ubiquitination or 20S peptidase activity (*9*), indicating they did not inhibit E1 or act as conventional proteasome inhibitors.

To understand how class IIB compounds inhibited proteolysis, we turned to a reconstituted system using purified 26S proteasomes and ubiquitinated Sic1 (UbSic1) (*10*). Degradation of Sic1 requires its ubiquitination by the ligase SCF^{Cdc4} (*11*, *12*), after which UbSic1 is docked to the 19S regulatory particle by a multi-Ub chain receptor (*13*). Proteolysis of UbSic1 requires removal of the multi-Ub chain, catalyzed by the metalloisopeptidase Rpn11 (*14*, *15*). The deubiquitinated substrate is concomitantly translocated into the 20S core particle, where it is degraded. Two class IIB molecules, C92 and C59 (Fig. 1D), strongly inhibited UbSic1 turnover in the reconstituted system (Fig. 1A). To address whether these

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Table 1. Characterization of compounds in *Xenopus* extract assays. Results are reported as percent inhibition (percent stimulation). Compounds (200 μM, except C10 and C92, tested at 100 μM) and cyclin-luciferase (cyc-luc) were added to interphase extracts and then induced to enter mitosis by addition of nondegradable cyclin B, or extracts were pretreated with nondegradable cyclin B to allow entry into mitosis before addition of test compound and cyc-luc. Cdh1 was added to interphase extracts before addition of compound and cyc-luc. Interphase extracts were treated with recombinant axin to induce turnover of β-catenin-luciferase. Parentheses indicate those values where stimulation, rather than inhibition, was observed by addition of compound to the reaction.

Compound	Addition before mitotic entry	Addition after mitotic entry	Cdh1-activated interphase extract	β-catenin reporter protein
		Class IA		
C77	100	4	(12)	0
C58	100	5	(8)	2
C82	100	0	0	0
C34	100	0	(8)	6
C62	84	0	(8)	0
C61	77	8	(8)	2
C13	75	0	(9)	0
C18	73	4	(7)	0
C25	66	3	(6)	0
C54	54	3	(6)	0
C67	53	3	(8)	3
C40	42	0	(6)	3
		Class IB		
C39	100	9	(7)	67
C57	100	4	0	60
C51	100	0	0	30
C10	33	0	(4)	21
		Class IIA		
C1	100	100	35	6
C2	80	50	100	0
C8	70	63	20	0
		Class IIB		
C23	100	100	100	27
C59	97	100	100	70
C92	60	22	65	21

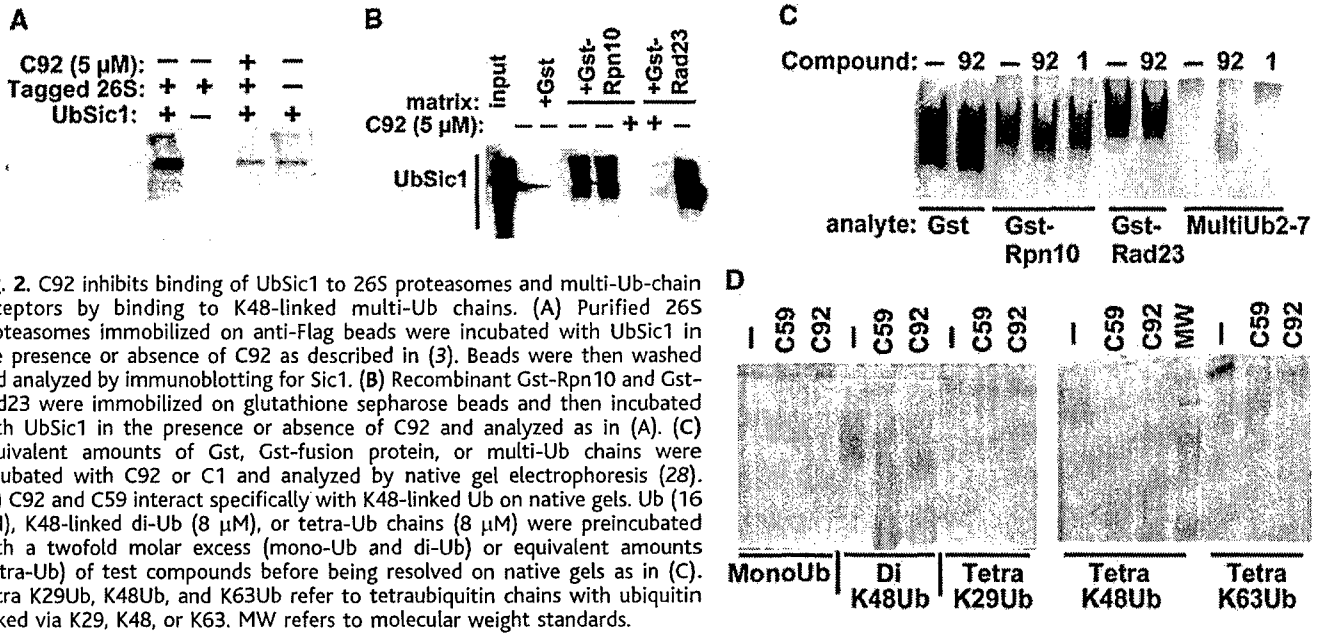

Fig. 2. C92 inhibits binding of UbSic1 to 26S proteasomes and multi-Ub-chain receptors by binding to K48-linked multi-Ub chains. (A) Purified 26S proteasomes immobilized on anti-Flag beads were incubated with UbSic1 in the presence or absence of C92 as described in (3). Beads were then washed and analyzed by immunoblotting for Sic1. (B) Recombinant Gst-Rpn10 and Gst-Rad23 were immobilized on glutathione sepharose beads and then incubated with UbSic1 in the presence or absence of C92 and analyzed as in (A). (C) Equivalent amounts of Gst, Gst-fusion protein, or multi-Ub chains were incubated with C92 or C1 and analyzed by native gel electrophoresis (28). (D) C92 and C59 interact specifically with K48-linked Ub on native gels. Ub (16 μM), K48-linked di-Ub (8 μM), or tetra-Ub chains (8 μM) were preincubated with a twofold molar excess (mono-Ub and di-Ub) or equivalent amounts (tetra-Ub) of test compounds before being resolved on native gels as in (C). Tetra K29Ub, K48Ub, and K63Ub refer to tetraubiquitin chains with ubiquitin linked via K29, K48, or K63. MW refers to molecular weight standards.

compounds acted upstream or downstream of Rpn11 isopeptidase, we treated proteasomes with the 20S inhibitor epoxomicin, which results in Rpn11-dependent substrate deubiquitination (*14*, *16*) and accumulation of deubiquitinated Sic1 within the 20S chamber (*13*). This reaction was completely blocked by C92 (Fig. 1B), with a median inhibitory concentration (IC_{50}) of about 400 nM (Fig. 1C). C59, which is structurally related to C92, also inhibited deubiquitination of UbSic1 (IC_{50} = 1 μM), whereas C23 inhibited marginally (fig. S5). Thus C92 and C59 potently blocked proteolysis at or upstream of the essential isopeptidase-dependent step.

Selective recognition of the multi-Ub chain by the 26S proteasome is the first step in UbSic1 degradation (*13*). C92 strongly inhibited binding of UbSic1 to purified 26S proteasomes (Fig. 2A), suggesting that it inhibited UbSic1 turnover by blocking the first step in the degradation process. The multi-Ub chain receptors Rad23 and Rpn10 serve a redundant role in targeting UbSic1 to the proteasome and sustaining its degradation (*13*). In the absence of the Ub-binding activities of Rpn10 and Rad23, UbSic1 is not recruited, deubiquitinated, or degraded by purified 26S proteasomes. We thus tested whether C92 could interfere with binding of UbSic1 to recombinant Rpn10 and Rad23. C92 abolished binding of UbSic1 to both proteins (Fig. 2B), even though these receptors use distinct domains [the Ub-interaction motif (UIM) and the Ub-associated (UBA) domain, respectively] to bind ubiquitin chains (*17*). C59 also abrogated binding of UbSic1 to Rpn10, whereas other compounds were without effect (fig. S5).

To distinguish whether C92 inhibited proteolysis by binding to proteasome receptor proteins or to the Ub chain on Sic1, we exploited the negative charge of C92 to determine whether compound binding induced a mobility shift of the target proteins upon fractionation on a native polyacrylamide gel. C92 was preincubated with recombinant Rpn10, Rad23, or a mixture of Ub chains containing two to seven Ub molecules. The mobility of the multi-Ub chains, but not Gst-Rpn10 or Gst-Rad23, was altered by incubation with C92, suggesting that C92 bound Ub chains (Fig. 2C). Ubiquitin molecules can be linked to each other in vivo through different internal lysines, including K29, K48, and K63 (*18*). The K48-linked chain is the principal targeting signal in proteolysis, whereas K63-linked chains are implicated in enzyme regulation (*19*). Whereas C92 and C59 efficiently shifted the native gel mobility of K48-linked ubiquitin chains, they had little or no effect on K29- or K63-linked chains (Fig. 2D). Because C92 and C59 bind to ubiquitin chains and block interactions with proteasome-associated receptors without affecting 26S assembly or peptidase activity (fig. S6), we refer to these compounds as ubistatin A and B, respectively.

We next tested the ability of ubistatins to block proteolysis of ornithine decarboxylase (ODC), whose degradation does not require ubiquitin (*20*). Whereas a 30-fold molar excess of ubistatin A over the substrate strongly inhibited UbSic1 degradation by purified yeast proteasomes (Fig. 1A), a 100-fold molar excess of ubistatin A over the substrate had no effect on degradation of radiolabeled ODC by purified rat proteasomes (fig. S7). Ubistatin B marginally inhibited ODC turnover at this concentration (12%). In contrast, a 20-fold molar excess of cold ODC inhibited degradation of labeled ODC by 43% under the same conditions. These data indicate that ubistatins at low concentrations preferentially inhibit the degradation of ubiquitin-dependent substrates. Inhibition of ODC turnover by high concentrations of ubistatins, especially ubistatin B (fig. S7), may reflect either nonspecific activity or specific inhibition of a targeting mechanism shared by ubiquitin-dependent and ubiquitin-independent substrates of the proteasome (*20*).

On the basis of the selectivity of ubistatin A for binding K48-linked chains and inhibiting the ubiquitin-dependent turnover of Sic1 but not the ubiquitin-independent turnover of ODC, we tested the effect of ubistatin A on protein degradation within intact mammalian cells. Because the negative charge on ubistatin A precluded efficient membrane permeation, we introduced the compound into cells by microinjection and monitored degradation of an androgen receptor–green fluorescent protein (AR-GFP) fusion protein by fluorescence microscopy. Microinjection of a synthetic compound (protac, proteolysis-targeting chimeric molecule), which recruits AR-GFP to $SCF^{\beta\text{-}TRCP}$, induces rapid proteasome-dependent turnover of AR-GFP (*21*). Microinjection of 100 nM ubistatin A into mammalian cells inhibited the Protac-induced degradation of AR-GFP as efficiently as 100 nM epoxomicin (fig. S8), demonstrating that ubistatin A is an effective

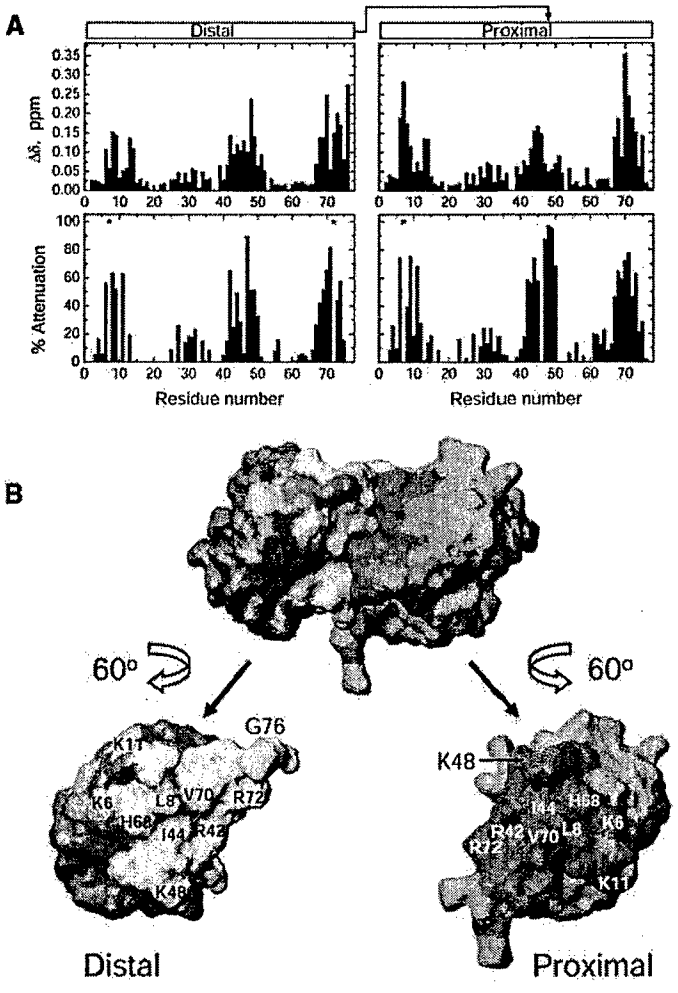

Fig. 3. Ubistatin A binding to K48-linked di-Ub induces site-specific perturbations in NMR spectra for both Ub domains. (**A**) Backbone NH chemical shift perturbation, Δδ, and percent signal attenuation caused by ubistatin A binding as a function of residue number for the distal (left) and the proximal (right) domains. Ub units are called "distal" and "proximal" to reflect their location in the chain relative to the free C terminus. The diagram (top) depicts the location of the G76-K48 isopeptide bond between the two Ub domains. Asterisks indicate residues that showed significant signal attenuation that could not be accurately quantified because of signal overlap. (**B**) Mapping of the perturbed sites on the surface of di-Ub. The distal and proximal domains are shown in surface representation and colored blue and green, respectively; the perturbed sites on these domains are colored yellow and red and correspond to residues with Δδ > 0.075 parts per million and/or signal attenuation greater than 50%. Numbers indicate surface location of the hydrophobic patch and some basic residues along with G76 (distal) and the side chain of K48 (proximal).

inhibitor of ubiquitin-dependent degradation in multiple experimental settings.

The specificity of ubistatin A for K48-linked ubiquitin chains suggested that it might bind at the Ub-Ub interface, which is well defined in K48-linked chains but is not present in K63-linked di-ubiquitin (Ub_2) (*22*). We performed nuclear magnetic resonance (NMR) titration studies of K48-linked Ub_2 by using a segmental labeling strategy (*23*). Well-defined site-specific perturbations were observed in the resonances of the backbone amides of both Ub units in Ub_2 (Fig. 3), indicating that the hydrophobic patch residues L8, I44, V70 (*24*), and neighboring sites (including basic residues K6, K11, R42, H68, and R72) experienced alterations in their molecular environment upon binding of ubistatin A. The same hydrophobic patch is involved in the formation of the interdomain interface in Ub_2 (*23*, *25*) and mediates the binding of ubiquitin to multiple proteins containing CUE (coupling of ubiquitin conjugation to ER degradation), UBA, and UIM domains (*17*). At the high concentrations of compound used in the NMR titration experiments, ubistatin A induced a similar pattern of chemical shift perturbations in monomeric ubiquitin, suggesting that the effect of ubistatin A on Ub_2 arises from its direct binding to the hydrophobic patch and the basic residues around it. The same sites are perturbed when ubistatin A binds tetra-Ub chains (*26*).

Although there is intense interest in developing drugs for defined molecular targets, it is often difficult to know a priori which proteins can be most effectively targeted with small molecules. Our study demonstrates that chemical genetic screens in complex biochemical systems such as *Xenopus* extracts can identify small-molecule inhibitors that act through unexpected mechanisms. Although target identification remains challenging, our work highlights the value of reconstituted biochemical systems to illuminate the mechanism of action of inhibitors discovered in unbiased screens. The recent approval of the 20S proteasome inhibitor Velcade (Millenium Pharmaceuticals, Cambridge, MA) for treatment of relapsed multiple myeloma (*27*) has suggested that the ubiquitin-proteasome system is an attractive target for cancer drug development. The identification of ubistatins indicates that the ubiquitin chain itself provides another potential opportunity for pharmacological intervention in this important pathway.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/306/5693/117/DC1

Materials and Methods
Figs. S1 to S8
Table S1

1 June 2004; accepted 6 August 2004

Regulation of Cytokine Receptors by Golgi N-Glycan Processing and Endocytosis

Emily A. Partridge,[1,3] Christine Le Roy,[1] Gianni M. Di Guglielmo,[1] Judy Pawling,[1] Pam Cheung,[1,2] Maria Granovsky,[1,2] Ivan R. Nabi,[4] Jeffrey L. Wrana,[1,2] James W. Dennis[1,2,3]*

The Golgi enzyme β1,6 N-acetylglucosaminyltransferase V (Mgat5) is upregulated in carcinomas and promotes the substitution of N-glycan with poly N-acetyllactosamine, the preferred ligand for galectin-3 (Gal-3). Here, we report that expression of Mgat5 sensitized mouse cells to multiple cytokines. Gal-3 cross-linked Mgat5-modified N-glycans on epidermal growth factor and transforming growth factor–β receptors at the cell surface and delayed their removal by constitutive endocytosis. Mgat5 expression in mammary carcinoma was rate limiting for cytokine signaling and consequently for epithelial-mesenchymal transition, cell motility, and tumor metastasis. Mgat5 also promoted cytokine-mediated leukocyte signaling, phagocytosis, and extravasation in vivo. Thus, conditional regulation of N-glycan processing drives synchronous modification of cytokine receptors, which balances their surface retention against loss via endocytosis.

Co-translational modification of proteins in the endoplasmic reticulum by N-glycosylation facilitates their folding and is essential in single-cell eukaryotes. Metazoans have additional Golgi enzymes that trim and remodel the N-glycans, producing complex-type N-glycans on glycoproteins destined for the cell surface. Mammalian development requires complex-type N-glycans containing N-acetyllactosamine antennae, because their complete absence in Mgat1-deficient embryos is lethal (*1*, *2*). Deficiencies in N-acetylglucosaminyltransferase II and V (Mgat2 and Mgat5) acting downstream of Mgat1 reduce the content of N-acetyllactosamine, and mutations in these loci result in viable mice with a number of tissue defects (*3*, *4*). N-glycan processing generates ligands for various mammalian lectins, but the consequences of these interactions are poorly understood. The galectin family of N-acetyllactosamine-binding lectins has been implicated in cell

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RESEARCH INTERESTS

Growth Factor Signal Transduction
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1985-1986 Internship, Pediatrics, Children's Hospital of Los Angeles

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1994	Young Investigator Award in Oncology, American Society for Pediatric Hematology-Oncology
1995	UCLA Frontiers of Science Award
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1998-2003	Leukemia Society of America Scholar Award
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2002	Full member, Molecular Biology Institute, UCLA
2003-present	Children's Oncology Group, Myeloid Biology Subcommittee
2003-2007	Member, Grant Review Subcommittee on Leukemia, Immunology, and Blood Cell Development for American Cancer Society
2004	NIH Study Sections on Drug Discovery and Molecular Pharmacology and Basic Mechanisms of Cancer Therapy, and Special Emphasis Panel on Diamond-Blackfan Anemia and Bone Marrow Failure syndromes
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2004	Moderator, Leukemia Session at American Society for Pediatric Hematology-Oncology Annual Meeting
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2005	"Ask the Experts" in Pediatric Cancer, AACR Public Forum, Anaheim, CA.
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2005-present	Member, Translation Research Program Review Subcommittee for the Leukemia and Lymphoma Society of America
2005	Moderator, Pediatric Hematology-Oncology session, PAS/ASPHO meeting, Washington DC, May 2005

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1994-present	UCLA Cancer Committee
1998-1999	Chair of Tumor Cell Biology ACCESS Affinity Group for Graduate Students
1996-2002	Western Society for Pediatric Research (WSPR) Council member
2002-present	Search Committee for Pediatric Pulmonary
2002-present	Search Committee for Pediatric Nephrology
2002-present	Search Committee for Pediatric Hematology-Oncology

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1994-present	Faculty Mentor on the Medical Student Training Program
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1995-present	Principal Investigator on the UCLA ACCESS program for graduate Students

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1993-present	Pediatric Hematology-Oncology elective
1993-present	Advanced Clinical Clerkship in Pediatric Hematology-Oncology
1993-present	Laboratory course in Biochemistry for first year medical students
1993-present	Pediatric Clerkship
1993-present	Advanced Clinical Clerkship in Pediatrics
1995	Ethics and Accountability in Biomedical Research
1995-1997	Major Concepts in Oncology
1995	Molecular and Cellular Foundations of Disease
1993-1997	Organization of Pediatric Hematology-Oncology weekly clinic conferences
1995-1999	Organization of the Pediatric Departmental Monthly Research Seminars

1999-present	M229 Course on Cell Biology and Pathogenesis for ACCESS Graduate Students on "Cell Cycle" (organized by Patricia Johnson)
1996-2003	Pathophysiology Course in Hematopathology (session on Lymphoma)
2005-present	Co-Director of the Signal Transduction Program Area, Jonsson Comprehensive Cancer Center
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Clinical Activities

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GRANTS

1989-1990 American Cancer Society Clinical Oncology Fellowship

1990-1993 5 F32 CA08974-04 Individual National Research Service Award Molecular Analysis of Target Cell Response to Human GM-CSF ($102,100); National Cancer Institute (Judith Gasson, Ph.D., P.I.)

1996-2002 Fellowship Award, Molecular Characterization of GM-CSF Action ($70,000) Leukemia Society of America (Judith C. Gasson, Ph.D., P.I.)

1993-1998 K08 CA59463, Clinical Investigator Award, Molecular Characterization of GM-CSF Action ($383,400), National Cancer Institute (Judith Gasson, Ph.D. P.I.)

1993-1996 3017-93, Special Fellow Award, Molecular Analysis of GM-CSF Action ($100,400), Leukemia Society of America (K. Sakamoto, M.D., P.I.)

1992-1995 Career Development Award, Molecular Characterization of GM-CSF Action ($150,000), STOP CANCER (K. Sakamoto, M.D., P.I.)

1992-1993 Seed Grant, Mutation Analysis of Structure-Function Relationships of Human GM-CSF Receptor Beta Subunit ($30,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)

1992-1993 Mutation Analysis of Structure-Function Relationships of the Human GM-CSF Receptor Beta Subunit ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (declined) (K. Sakamoto, M.D., P.I.)

1993-1995 Molecular Regulation of egr-1 by IL-3 and PIXY321 in Myeloid Leukemias ($100,000), Concern II (K. Sakamoto, M.D., P.I.)

1994 The Role of Cyclins in Myeloid Leukemias ($25,000), Southern California Children's Cancer Service and Couples Against Leukemia (K. Sakamoto, P.I.)

1995 UCLA Academic Senate Award ($1,500), "Stem Cell Factor Activation of Signal Transduction in Myeloid Leukemic Cells" (K. Sakamoto, M.D., P.I.)

1995 UCLA Frontiers of Science Award, The Regulation and Functional Role of p55CDC in Myeloid Leukemias ($28,000) (K. Sakamoto, M.D., P.I.)

1995 UCLA Prime Faculty Research Award, Molecular Regulation of Myeloid Cell Differentiation ($25,000) (K. Sakamoto, M.D., P.I.)

1995 Seed Grant, The Role of SRE-Binding Proteins During Signal Transduction in Myeloid Leukemias ($27,000), Jonsson Comprehensive Cancer Center (K. Sakamoto, M.D., P.I.)

1995 New Assistant Professor Grant, Transcriptional Regulation of egr-1 by Stem Cell Factor in Myeloid Leukemias ($35,000), Cancer Research Coordinating Committee (K. Sakamoto, M.D., P.I.)

1995-1997 Shannon Award, NIH (NCI) 1R55CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($80,000) (K. Sakamoto, M.D., P.I.)

1996 Concern II Foundation; Molecular Analysis of IL-3 and PIXY321 Signaling Pathways in Myeloid Leukemias ($50,000) (K. Sakamoto, M.D., P.I.)

1996-2002 First Award R29CA68221, Molecular Regulation of Myeloid Cell Differentiation, ($350,000), NIH/NCI (K. Sakamoto, M.D., P.I.)

7/97-6/99 UC Biotechnology STAR Project, S97-03 "p55Cdc and Cell Cycle Regulation" ($40,000); Amgen, Inc. and University of California (K. Sakamoto, M.D., P.I.)

7/98-6/99 Contract with Eli Lilly, Inc. "Multiple Resistance Genes in Leukemias" ($32, 000), Co-PI with Leonard Rome, Ph.D. (K. Sakamoto, M.D., P.I.)

7/98-6/99 Jonsson Comprehensive Cancer Center Seed Grant, "Use of Low Molecular Weight Heparin in Cancer Patients Receiving Stem Cell Transplants," ($30,000), Co-P.I. with Dr. Sinisa Dovat, M.D. (fellow)

7/98-6/2003 Leukemia Society of America Scholar Award, 1497-99 "The Role of p55Cdc during Myelopoiesis" ($350,000), Leukemia Society of America (K. Sakamoto, P.I.)

1/99-12/2001 Investigator initiated grant, California Cancer Research Program, "Cell Cycle Control and Cancer" ($400,000), California Department of Health Services (K. Sakamoto, P.I.)

7/99- 6/2000 Jonsson Comprehensive Cancer Center Seed Grant, "Development of a Novel Class of Protein-inhibiting Anti-cancer Therapeutics" ($15,000), K. Sakamoto (P.I.) and Raymond Deshaies (Co-P.I., Caltech)

1/2000 CaPCURE research award, "Development of a Novel Class of Protein-Inhibiting Therapeutics for Prostate Cancer" ($100,000). Raymond Deshaies (P.I., Caltech), K. Sakamoto, and Craig Crews (Co-P.I., Yale University).

1/99-12/02 Research Project Grant, "Molecular Analysis of Myeloid Cell Proliferation" ($300,000); American Cancer Society (K. Sakamoto, P.I.)

8/01-7/03 UC Biostar, "Targeting the estrogen receptor for Proteolysis", with Celgene, Inc. ($40,000), K Sakamoto, P.I.

1/02-12/02 CaPCURE research award, "Targeting the Androgen Receptor for Degradation in Prostate Cancer" ($75,000) K.Sakamoto (P.I.), Raymond Deshaies (Co-P.I., Caltech) and Craig Crews (Co-P.I., Yale University).

6/02-7/03 National Cancer Coalition, "Signal Transduction and Cell Cycle Analysis in Leukemia" ($5,000), K. Sakamoto (P.I.).

1/03-12/06 American Cancer Society, Research Scholar Award. "The role of CREB in Leukemogenesis," ($625,000). K. Sakamoto (P.I.).

1/03-6/04 Department of Defense, "Targeting the estrogen receptor for ubiquitination and proteolysis in breast cancer," ($222,819). K. Sakamoto (P.I.)

1/03-12/03 Diamond-Blackfan Anemia Foundation, "AML in Diamond-Blackfan Anemia: Molecular Basis and Therapeutic Strategies," ($25,000). K. Sakamoto (P.I.)

1/1/03-12/31/04 SPORE grant in Prostate Cancer Research, Seed Grant Award, "Targeting the Androgen Receptor for proteolysis in Prostate Cancer," $75,000. K. Sakamoto (P.I.)

4/1/03-3/31/04 Stein-Oppenheimer Award, "Targeting the Estrogen Receptor in Breast Cancer," $20,000. K. Sakamoto (P.I.)

6/1/03-5/30/04 Genomic Exploration Seed Grant, Jonsson Comprehensive Cancer Center, "CREB and Human Leukemias," $5,000, K. Sakamoto (P.I.)

7/1/03-6/30/04 Susan G. Komen Breast Cancer Thesis Dissertation Award," $20,000. K. Sakamoto, R. J. Deshaies (P.I.)

1/04-12/07 NIH/NHLBI R01 (HL 75826), "The Role of CREB in Leukemogenesis," ($200,000/year). K. Sakamoto (P.I.)

9/04-8/06 R21, "Ubiquitination and Degradation in Cancer Therapy," ($135,000/year). K. Sakamoto (P.I.)

7/04-7/05 Department of Defense, "Identification of small non-peptidic ligands that bind the $SCF^{beta\text{-}TRCP}$ ubiquitin ligase to target the ER for ubiquitination and degradation ($75,000). K. Sakamoto (P.I.)

7/05-5/07 Fulbright Fellowship/MEC (Spain) postdoctoral fellowship, "Targeting the Androgen Receptor for Ubiquitination and Degradation: A new strategy for Therapy in Prostate Cancer" ($60,000), K. Sakamoto and R. Deshaies (Co-P.I.).

5/05 Boyer/Parvin Postdoctoral Fellow Award ($5,000), awarded to Deepa Shankar, Ph.D., K. Sakamoto (P.I.)

7/05 Stone Research Award ($1,000) award to undergraduate student Winston Wu, K. Sakamoto (P.I.)

7/05-6/07 Department of Defense postdoctoral fellowship "Targeting the Androgen Receptor for Ubiquitination and Degradation: A New Strategy for Therapy in Prostate Cancer."

TRAINING FACULTY ON THE FOLLOWING TRAINING GRANTS (NIH T32 and K12 Programs)

Tumor Cell Biology
Tumor Immunology
Human and Molecular Development
Hematology
Vascular Biology
Neonatology
Medical Scientist Training Program (MSTP)

TRAINEES

1991-1993	Hu-Jung Julie Lee, undergraduate student
1992-1993	Elana Lehman, medical student
1993-present	Kathy Hwain Shin, undergraduate student, Work/study and Lab Assistant
1994-1995	Robert C. Mignacca, M.D., postdoctoral fellow
1994-1995	Stephen Phillips, undergraduate student, Student Research Project

1995	Allison Wong, medical student; Short Term Training Program; Recipient of Howard Hughes NIH Research Scholar Award, 1996-1997
1995	Ramona Rodriguez, medical student; Short Term Training Program, Centers of Excellence
1995-2000	Evelyn Kwon, graduate student
1996	Michael Mendoza, medical student, Short Term Training Program; Centers of Excellence and FIRST/STAR Award recipient
1996-2002	Patricia Mora-Garcia (awarded Minority Supplement Award from NIH/NCI), Dept. Pathology and Laboratory Medicine
1996-2002	Michael Lin, graduate students (recipient of NIH/NCI Tumor Cell Biology Training Grant), Dept. Pathology and Laboratory Medicine
1997	Raymond Wang, medical student, Short Term Training Program
1995-1999	Wayne Chu, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of 1999 Merle Carson Lectureship, 1st Prize Southwestern Pediatric Society, The Tenth Joseph St. Geme, Jr. Research Award for UCLA Pediatric Trainees)
1999-2000	Kristin Baird, M.D. Pediatric Resident, Mattel Children's Hospital at UCLA, research elective
2000-present	Deepa Shankar, Ph.D., Postdoctoral fellow (NIH Tumor Cell Biology Postdoctoral fellowship, JCCC fellowship).
2001-2002	Heather Crans, graduate student (recipient of NIH Tumor Immunology Training Grant), Dept. Pathology and Laboratory Medicine
2001-2003	Athena Countouriotis, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA, research elective (recipient of Resident Research Award, American Academy of Pediatrics)
2002-present	Jerry Cheng, M.D., Pediatric Resident, Mattel Children's Hospital at UCLA (won SPR House Officer Award 2003, ASPHO/SPR meeting, Seattle, WA).
2002-2003	Tamara Greene, Medical Student, UCLA School of Medicine
2002-2003	Johnny Chang, M.D., Medical Oncology Fellow, Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine (recipient Of NIH Hematology Training Grant)
2003-present	Noah Federman, M.D., Pediatric Resident, Mattel Children's Hospital, research elective
2003	Andy Liu, undergraduate student (Recipient of Undergraduate scholarship award)
2003	Ryan Stevenson, undergraduate student
2004	Maricela Rodriguez, medical student
2005	Jenny Hernandez, Saul Priceman, Jose Cordero, Gloria Gonzales, Salemiz Sandoval
	2005 Cid Sumolong, STTP, UCLA medical student
2005-present	Winston Wu, undergraduate (recipient of John Stone Award)
2005-present	Salemiz Sandoval, graduate student.

BIBLIOGRAPHY

PEER-REVIEWED

1. Nagahashi, G and Hiraike (**Sakamoto**) KM. Effect of centrifugation time on sedimentation of plant organelles. Plant Physiol 69:546-548, 1982.

2. Yamamoto J, Yap J, Hatakeyama J, Hatanaka H, Hiraike (**Sakamoto**) **K**, Wong L: Treating Asian Americans in Los Angeles. Psychiatry 8:411-416, 1985.

3. **Sakamoto KM**, Bardeleben C, Yates KE, Raines MA, Golde DW, Gasson JC: 5' upstream sequence and genomic structure of the human primary response gene, EGR-1/TIS8. Oncogene 6:867-871, 1991.

4. **Sakamoto KM**, Nimer SD, Rosenblatt JD, Gasson JC: HTLV-I and HTLV-II tax *trans*-activate the human EGR-1 promoter through different *cis*-acting sequences. Oncogene 7:2125-2130, 1992.

5. **Sakamoto-K**, Erdreich Epstein A, deClerck Y, Coates T: Prolonged clinical response to vincristine treatment in two patients with idiopathic hypereosinophilic syndrome. Am J Ped Hemat Oncol 14:348-351, 1992.

6. **Sakamoto KM**, Fraser JK, Lee H-J J, Lehman E, Gasson JC: GM-CSF and IL-3 signaling pathways converge on the CREB-binding site in the human EGR-1 promoter. Mol Cell Biol, 14: 5920-5928, 1994.

7. Lee H-J J, Mignacca RM, and **KM Sakamoto**. Transcriptional activation of egr-1 by Granulocyte-Macrophage Colony-Stimulating Factor but not Interleukin-3 requires phosphorylation of CREB on Serine 133. J. Biol. Chem., 270: 15979-15983, 1995.

8. Wong A and **KM Sakamoto**. GM-CSF-Induces the Transcriptional Activation of Egr-1 Through a Protein Kinase A-Independent Signaling Pathway. J Biol Chem 270: 30271-30273, 1995.

9. Horie M, **Sakamoto KM,** Broxmeyer HC. Regulation of egr-1 gene expression by retinoic acid in a human growth factor-dependent cell line. Int J Hematology, 63: 303-309, 1996.

10. Mignacca RC, Lee H-J J, and **KM Sakamoto.** Mechanism of Transcriptional Activation of the Immediate Early Gene Egr-1 in response to PIXY321. Blood, 88: 848-854, 1996.

11. Kao CT, Lin M, O'Shea-Greenfield A, Weinstein J, and **KM Sakamoto.** p55Cdc Overexpression Inhibits Granulocyte Differentiation Through an Apoptotic Pathway. Oncogene, 13:1221-1229, 1996.

12. Kwon EM and **KM Sakamoto**. Molecular Biology of Myeloid Growth Factors. J Inv Med, 44: (8) 442-445 October, 1996.

13. Watanabe S, Kubota H, **Sakamoto KM**, and K Arai. Characterization of cis-acting sequences and trans-acting signals regulating early growth response gene 1 (egr-1) promoter through granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells. Blood, 89:1197-1206, 1997.

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17. Chu Y-W, Wang R, Schmid I and **Sakamoto KM**. Analysis of Green Fluorescent Protein with Flow Cytometry in Leukemic Cells. Cytometry, 333-339, 1999.

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19.Kwon EM, Raines MA and **KM Sakamoto**. GM-CSF Induces CREB Phosphorylation Through Activation of pp90Rsk. Blood, 95: 2552-2558, 2000.

20.Mora-Garcia PM and **KM Sakamoto**. Potential Role of SRF and Fli-1 in G-CSF-induced Egr-1 Gene Expression. J Biol Chem, 275: 22418-22426, 2000.

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22. Wong A, **KM Sakamoto**, and EE Johnson. Differentiating Osteomyelitis and Bone infarctions in sickle cell patients. Ped Em Care, 17:60-66, 2001.

23. Lin M and **KM Sakamoto.** p55Cdc/Cdc20 Overexpression Promotes Early G1/S Transition in Myeloid Cells. Stem Cells19: 205-211, 2001.

24. Shou W, **Sakamoto KM**, Keener J, Morimoto KW, Hoppe GJ, Azzam R, Traverso EE, Feldman RFR, DeModena J, Charbonneau H, Moazed D, Nomura M and RJ Deshaies. RENT complex stimulates RNA Pol I transcription and regulates nucleolar structure independently of controlling mitotic exit. Mol Cell, 8: 45-55, 2001.

25. **Sakamoto KM**, Crews CC, Kim KB, Kumagai A, Mercurio F, and RJ Deshaies. Protac: A Chimeric Molecule that targets Proteins to the SCF for Ubiquitination and Degradation. Proc Natl Acad Sci USA, 98: 8554-8559, 2001.

26. Gubina E, Luo X, Kwon EM, **Sakamoto KM**, Shi YF and RA Mufson. βc Receptor Cytokine Stimulation of CREB Transcription Factor Phosphorylation by Protein Kinase C: A Novel Cytokine Signal Transduction Cascade. J Immunol 167: 4303-4310, 2000.

27. Xu Z, Cziarski R, Wang Q, Swartz K, **KM Sakamoto**, and D Gupta. Bacterial peptidoglycan-induced TNF-α transcription is mediated through the transcription factors Egr-1, Elk-1, and NF-kB. J Immunol, 167: 6972-6985, 2001.

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29. Mora-Garcia P, Pan R, and **KM Sakamoto**. G-CSF Regulation of SRE-binding proteins in myeloid leukemia cells. Leukemia, 16: 2332-2333, 2002

31. Lin M, Chang JK, and **KM Sakamoto**. Regulation of the Cell cycle by p55CDC in myeloid cells. Exp Mol Path, 74: 123-8, 2003.

32. Mora-Garcia P, Cheng J, Crans-Vargas H, and **KM Sakamoto**. The role of SRE-binding proteins and CREB in Myelopoiesis., Stem Cells, 21: 123-130, 2003.

33. Hsu H, Rainov NG, Quinones A, Eling DJ, **Sakamoto KM**, and MA Spears. Combined radiation and cytochrome CYP4B1/4-ipomeanol gene therapy using the EGR1 promoter. Anticancer Res 23: 2723-2728, 2003.

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35. **Sakamoto KM**, Kim KB, Verma R, Ransick A, Stein B, and RJ Deshaies. Development of Protacs to Target Cancer-Promoting Proteins for Ubiquitination and Degradation. Mol Cell Proteomics, 12:1350-1358, 2003.

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37. Schneekloth JS, Fonseco F, Koldobskiy M, Mandal A, Deshaies RJ, **Sakamoto KM**, CM Crews. Chemical Genetic Control of Protein Levels: Selective *in vivo* Targeted Degradation. J Amer Chem Soc, 126(12); 3748-3754, 2004.

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39. Cheng JC, Esparza SD, Knez VM, **Sakamoto KM**, and TBMoore. Severe Lactic Acidosis in a 14-year old female with Metastatic Undifferentiated Carcinoma of Unknown Primary. Am J Ped Hem Onc, 26:780-782, 2004.

40. Mora-Garcia P, Wei J, and **KM Sakamoto**. G-CSF Induces Stabilization of Ets Protein Fli-1 During Myeloid Cell Development. Pediatr Res, 1:63-66, 2005.

41. Shankar D, Cheng J, Kinjo K, Wang J, Federman N, Gill A, Rao N, Moore TB, Landaw EM and **KM Sakamoto**. The role of CREB as a proto-oncogene in Hematopoiesis and in Acute Myeloid Leukemia. Cancer Cell, 7:351-362, 2005.

42. **Sakamoto KM.** Chimeric Molecules to Target Proteins for Ubiquitination and Degradation. Methods in Enzymology (Ubiquitin and Proteasome System), *in press*.

REVIEWS

1. **Sakamoto KM**, Golde DW, Gasson JC: The biology and clinical applications of granulocyte-macrophage colony-stimulating factor. J Peds 118:S17-S20, 1991.

2. **Sakamoto KM**, Gasson JC: Clinical applications of human granulocyte-macrophage colony-stimulating factor. Int J Cell Cloning 9:531-541, 1991.

3. **Sakamoto KM**, Mignacca RC, Gasson JC: Signal transduction by granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors. Receptors and Channels, 2: 175-181, 1994.

4. Mora-Garcia P and **KM Sakamoto**. Signal Transduction and Human Disease. Molecular Genetics and Metabolism, 66, 143-171, 1999.

5. Chu Y-W, Korb J and **KM Sakamoto**. Immune Thrombocytopenia Purpura. Pediatrics In Review, 21: 95-104, 2000.

6.**Sakamoto, KM**. Genetic and Functional Consequences of Cell Cycle Alteration in Cancer-AACR Special conference. 20-24 October 1999, San Diego, CA, USA. Idrugs 2000 3: 36-40.

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11. **Sakamoto KM**. Ubiquitin-dependent proteolysis: its role in human diseases and the design of therapeutic strategies. Mol Genet Metab 77:44-56, 2002.

12. **Sakamoto KM**. Targeting Ubiquitinylation for Drug Discovery. Meeting review, Idrugs 5: 779-781, 2002.

13. Cheng J, Moore TB, and **KM Sakamoto**. RNA interference and Human Disease. Mol Genet Metab, 80: 121-128, 2003.

14. Shankar D and **KM Sakamoto**. The Role of Cyclic-AMP Binding Protein (CREB) in leukemia cell proliferation and acute leukemias. Leuk Lymphoma, 45:265-70, 2004

15. **Sakamoto KM**. Knocking Down Human Disease: Potential uses of RNAi in Research and Gene Therapy. Pediatr Res, 55:912-913, 2004.

16. Cheng JC and **KM Sakamoto**. The Emerging Role of RNA Interference in the Design of Novel Therapeutics in Oncology (invited review), Cell Cycle, 3: 1398-1401, 2004.

17. **Sakamoto KM**. A Pediatric Approach to Classification of MDS. Highlights of the American Society of Pediatric Hematology-Oncology 17th Annual Meeting, May 2004. Medscape from WebMD.

18. **Sakamoto KM**. Understanding the Pathophysiology of Marrow Failure in MDS. Highlights of the American Society of Pediatric Hematology-Oncology 17th Annual Meeting, May 2004. Medscape from WebMD.

19. **Sakamoto KM**. Clinical Aspects of Childhood MDS. Highlights of the American Society of Pediatric Hematology-Oncology 17th Annual Meeting, May 2004. Medscape from WebMD.

20. Esparza SD and **KM Sakamoto**. Topics in Pediatric Leukemia-Acute Lymphoblastic Leukemia. MedGenMed Hematology-Oncology, published online on Medscape from WebMD, 2005.

21. Casillas J and **KM Sakamoto**. Topics in Pediatric Leukemia-Acute Lymphoblastic Leukemia and Late Effects in Long-Term Survivors. MedGenMed Hematology-Oncology, published online on Medscape from WebMD, 2005.

22. Lasky J and **KM Sakamoto**. Topics in Pediatric Leukemia-Myelodysplastic and Myeloproliferative Disorders of Childhood. MedGenMed Hematology-Oncology, published online on Medscape from WebMD, 2005.

23. Cheng JC and **KM Sakamoto**. Topics in Pediatric Leukemia-Acute Myeloid Leukemia. MedGenMed Hematology-Oncology, published online on Medscape from WebMD, 2005.

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25. Federman N and **KM Sakamoto**. Topics in Pediatric Leukemia-Fanconi's Anemia: New Insights. MedGenMed Hematology-Oncology, published online on Medscape from WebMD, 2005.

26. Shankar D, Cheng JC, and **KM Sakamoto**. The Role of Cyclic AMP Response Element Binding Protein in Human Leukemias. Cancer, *in press*.

27. Shankar D, Cheng JC, and **KM Sakamoto**. CREB as a Proto-oncogene in Hematopoiesis. Cell Cycle, *in press*.

28. Federman N and **KM Sakamoto**. The Genetic Basis of Bone Marrow Failure Syndromes in Children. Mol Gen Metab, *in press*.

BOOK CHAPTERS

1. Gasson JC, Baldwin GC, **Sakamoto KM**, DiPersio JF: The biology of human granulocyte-macrophage colony-stimulating factor (GM-CSF). In The Biology of Hematopoiesis, Dainiak N, Cronkite EP, McCaffrey R, Shadduck RK (eds). John Wiley & Sons, New York, 1990, pp. 375-384.

2. Schmid I and **KM Sakamoto,** Analysis of DNA Content and Green Fluorescent Protein Expression. Current Protocols in Flow Cytometry, 7.16.1-7.16.10, 2001.

3.Baird K and **KM Sakamoto**. Polycythemia. Manuscript (online) for Textbook in Pediatrics, emedicine.com.

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ABSTRACTS

1. **Sakamoto K**, Raines MB, Bardeleben C, Gasson JC: GM-CSF and TPA induce a subset of primary response TIS genes in both proliferating and terminally differentiated myeloid cells. American Society of Hematology; November, 1989. Blood 74(7):73a.

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4. **Sakamoto K**, Rosenblatt J, Gasson JC: *Trans*-activation of the human EGR-1 promoter by the HTLV-I and -II Tax proteins in T-cell lines. Blood 78:266a, 1991.^

5. Gilliland DG, Perrin S, **Sakamoto KM**, Bunn HF: Analysis of EGR-1 and other relevant genes in patients with 5q- syndrome. Blood 78:333a, 1991.

6. **Sakamoto KM**, Lee J, Gasson JC: GM-CSF-responsive sequences upstream of the primary response gene, EGR-1/TIS8, in a human factor-dependent myeloid leukemia cell line. J Cell Biochem 16C:M234, 1992.^

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8. **Sakamoto KM**, Lee JH-J, Gasson JC: GM-CSF and IL-3 mediate signal transduction through both overlapping and distinct upstream regulatory sequences in the human EGR-1 promoter. Blood 80:974, 1992.^

9. **Sakamoto KM**, Lee JH-J, Gasson JC: GM-CSF and IL-3 activate human early response gene (EGR-1) transcription through both overlapping and distinct upstream regulatory sequences. J Cell Biochem 17A:B974, 1993.

10. **Sakamoto KM**, Lee JH-J, Lehman ES, Gasson JC: GM-CSF and IL-3 induce early response gene expression through a novel transcription factor binding site. Blood 82:437a, 1993.^

11. Horie M, **Sakamoto KM**, Aronica S, Broxmeyer HE: Regulation of EGR-1 gene transcription by retinoic acid in a human factor-dependent cell line. Presented at the International Society of Experimental Hematology, 22:721; Minneapolis, MN; August 1994.

12. **Sakamoto KM**, Lee H-J J, Lehman ES, and Gasson JC: GM-CSF and IL-3 signal transduction in myeloid leukemic cells. Oral presentation and acceptance of the Young Investigator Award in Oncology, The American Society of Pediatric Hematology-Oncology, Chicago, IL; October 1994.^

13. **Sakamoto KM**, Lee H-J J, Lehman ES, and Gasson JC: GM-CSF and IL-3 Signal Transduction Pathways Converge on the Egr-1 and CREB-binding Sites in the Human egr-1 promoter. Presented to the American Association for Cancer Research meeting on Transcriptional Regulation of Cell Proliferation and Differentiation, Chatham, MA; October 1994.

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*oral presentation of abstract

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1. **Sakamoto KM.** "Cytokine Signals and Cell Cycle Control During Myelopoiesis" Childhood Leukemia, Biological and Therapeutic Advances. April 17, 1998, Los Angeles, California.

2. **Sakamoto KM.** Serine/Threonine Phosphorylation in Cytokine Signaling Workshop sponsored by the National Cancer Institute. March 30, 1999, Washington, D.C.

3. **Sakamoto KM.** "Signal Transduction Pathways Activated by GM-CSF." October 29-30, 1999. ACS Professors Meeting, New York.

4.**Sakamoto KM.** "Signal Transduction and Cell Cycle Control in Myeloid Cells" for Meet-the-Experts Breakfast, American Society of Hematology, December 5, 1999, New Orleans, LA.

5. **Sakamoto KM.** CapCURE meeting, September 2000, Lake Tahoe. "Novel Approach to treat Prostate Cancer"

6. **KM Sakamoto and RJ Deshaies.** What SCF Ubiquitin Ligases Are and how they can be used to regulate cancer progression, 4/01

7. **Sakamoto KM**. Bone marrow cells regenerate infracted myocardium, Journal Club, 4/01

8. **Sakamoto KM.** Acute Leukemia for Pediatric Residents at UCLA School of Medicine, 7/01

9. **Sakamoto KM.** ITP, Olive View Grand Rounds, 8/01

10. **Sakamoto KM.** Childhood Leukemia: causes and treatment. American Cancer Society, Los Angeles Chapter, 10/01

11. **Sakamoto KM.** "The Role of SCF Ubiquitin Ligase in Human Disease: Implications for Therapy." Caltech Biolunch, March 6, 2002.

12. **Sakamoto KM**. "Targeting Cancer-Promoting Proteins for Ubiquitination and Degradation" Signal Transduction Program Area Seminar, Jonsson Comprehensive Cancer Center, UCLA. August 1, 2003.

13. **Sakamoto KM.** "Development of Approaches to Target Proteins for Ubiquitination and Degradation in Human Disease." Thesis Defense, Caltech. December 18, 2003.

14. **Sakamoto KM.** "Role of CREB in Human Leukemias." Gene Medicine Seminar Series. Jan 26, 2004.

15. **Sakamoto KM**. "Childhood Neutropenias." Pediatric Resident Noon conference. February 4, 2004.

16. **Sakamoto KM**, "The Role of CREB in human leukemias", Gene Medicine Research Seminar, January 26th 2004.

17. **Sakamoto, KM**. "CREB and Acute Myeloid Leukemia," Leukemia Research Group Meeting, March 4, 2004.

18. **Sakamoto, KM.** "The Role of CREB in Leukemogenesis," Pediatric Research Seminar, May 20, 2004.

19. **Sakamoto, KM**. Meet the Professors lunch for UCLA ACCESS graduate students. October 6, 2004.

20. **Sakamoto KM**. UCLA Training Program in Tumor Biology. Retreat for UCLA ACCESS graduate students. October 31, 2004.

21. **Sakamoto KM**. "Hematology Jeopardy" Pediatric Noon Seminar, December 13, 2004.

21. **Sakamoto KM**. "Transcriptional Regulators in Normal and Malignant Hematopoiesis," MBI Noon Seminar, November 30, 2004.

22. **Sakamoto KM**. "Targeting Proteins for Ubiquitination and Degradation in Prostate Cancer" GU SPORE seminar, December 21, 2004.

23. **Sakamoto KM**. "Writing your first NIH grant: an overview," Pediatric Research Seminar, April 7, 2005.

24. **Sakamoto KM**. "Targeting the Ubiquitin-Proteasome System for Cancer Therapy." Minisymposium on Modulation of Protein Stability, AACR, Anaheim, CA, April 20, 2005.

25. **Sakamoto KM**. "The Role of CREB in Myelopoiesis." Myeloid Workshop, Annapolis, MD, 2005

26. **Sakamoto KM**. "The Use of RNA Interference to Study and Treat Human Disease." Cell Biology Methods workshop, PAS/SPR meeting, Washington, D.C., 2005.

27. **Sakamoto KM**. Introduction, Young Investigators Workshop. American Society of Pediatric Hematology-Oncology meeting, Washington D.C. 2005.

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[54] Chimeric Molecules to Target Proteins for Ubiquitination and Degradation

By KATHLEEN M. SAKAMOTO

Abstract

Protein degradation is one of the tactics used by the cell for irreversibly inactivating proteins. In eukaryotes, ATP-dependent protein degradation in the cytoplasm and nucleus is carried out by the 26S proteasome. Most proteins are targeted to the 26S proteasome by covalent attachment of a multiubiquitin chain. A key component of the enzyme cascade that results in attachment of the multiubiquitin chain to the target or labile protein is the ubiquitin ligase that controls the specificity of the ubiquitination reaction. Defects in ubiquitin-dependent proteolysis have been shown to result in a variety of human diseases, including cancer, neurodegenerative diseases, and metabolic disorders.

The SCF (Skp1-Cullin-F-box-Hrt1) complex is a heteromeric ubiquitin ligase that multiubiquitinates proteins important for signal transduction

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and cell cycle progression. A technology was developed known as Protac (*Pro*teolysis *Ta*rgeting Chimeric Molecule) that acts as a bridge, bringing together the SCF ubiquitin ligase with a protein target, resulting in its ubiquitination and degradation. The Protac contains an SCF-binding peptide moiety at one end that is recognized by SCF that is chemically linked to the binding partner or ligand of the target protein. The first demonstration of the efficacy of Protac technology was the successful recruitment, ubiquitination, and degradation of the protein methionine aminopeptidase-2 (MetAP-2) through a covalent interaction between MetAP-2 and Protac. Subsequently, we demonstrated that Protacs could effectively ubiquitinate and degrade cancer-promoting proteins (estrogen and androgen receptors) through noncovalent interactions *in vitro* and in cells. Finally, cell-permeable Protacs can also promote the degradation of proteins in cells. In this chapter, I describe experiments to test the ability of Protacs to target proteins *in vitro* and in cells.

Introduction

Ubiquitin-dependent proteolysis is a major pathway that regulates intracellular protein levels. Posttranslational modification of proteins by E3 ubiquitin ligases results in multiubiquitin chain formation and subsequent degradation by the 26S proteasome (Ciechanover *et al.*, 2000; Deshaies, 1999; Sakamoto, 2002). One potential approach to treating human disease is to recruit a disease-related protein to an E3 ligase for ubiquitination and subsequent degradation. To this end, a technology known as Protacs (*Pro*teolysis *Ta*rgeting Chimeric Molecules) was developed. The goal of Protac therapy is to create a "bridging molecule" that could link together a disease-related protein to an E3 ligase. Protacs consist of one moiety (e.g., a peptide), which is recognized by the E3 ligase. This moiety or peptide is then chemically linked to a binding partner of the target. The idea is that Protacs would bring the target to the E3 ligase in close enough proximity for multiubiquitin attachment, which would then be recognized by the 26S proteasome (Fig. 1). The advantage of this approach is that it is catalytic and theoretically can be used to recruit any protein, even those that exist in a multisubunit complex.

Several applications for Protac therapy are possible. In cancer, the predominant approach to treating patients is chemotherapy and radiation. Both of these forms of therapy result in complications because of effects on normal cells. Therefore, development of therapeutic approaches to specifically target cancer-causing proteins without affecting normal cells is desirable.

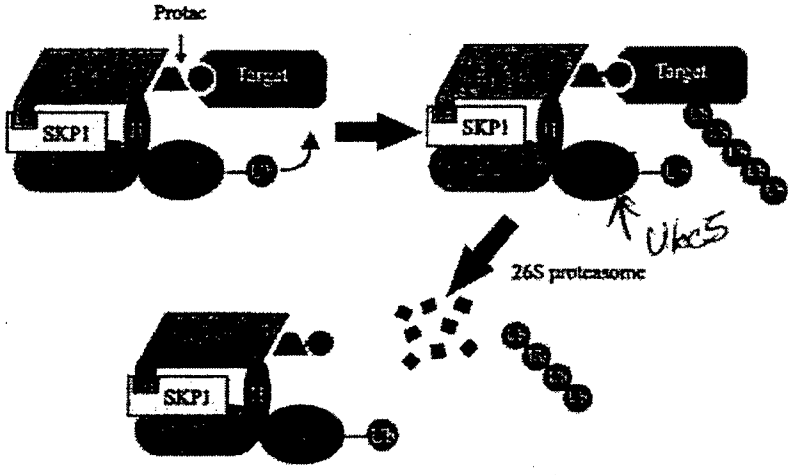


FIG. 1. Protac-1 targets MetAP-2 to SCF. Protac-1 is a chimeric molecule that consists of a phosphopeptide moiety and a small molecule moiety that interacts with the protein target (Sakamoto *et al.*, 2001) (See color insert.)

To test the efficacy of Protacs *in vitro* and *in vivo*, several components are essential. First, a functional E3 ligase is necessary, either in purified form or isolated from cell extracts. Additional components of ubiquitination reaction, including ATP, E1, E2, and ubiquitin, are also required. Second, a small peptide or molecule recognized by the E3 ligase must be identified. Finally, a target with a well-characterized binding partner must be selected that will be chemically linked to the peptide. Finally, successful application of Protacs technology depends on the ability of the Protac to enter cells to target the protein for ubiquitination and degradation. For clinical application, therapeutic drug concentrations are usually considered to be in the nanomolar range.

In addition to the use of Protacs for the treatment of human disease, these molecules provide a chemical genetic approach to "knocking down" proteins to study their function (Schneekloth *et al.*, 2004). The advantages of Protacs are that they are specific and do not require transfections or transduction. Protacs can be directly applied to cells or injected into animals without the use of vectors. Given the increased number of E3 ligases identified by the Human Genome Project, the possibilities for different combinations of Protacs that link specific targets to different ligases are unlimited. This chapter describes general strategies of testing the efficacy of Protacs using two E3 ligases as an example: $SCF^{\beta\text{-}TRCP}$ and Von Hippel

Lindau (VHL) complexes (Ivan *et al.*, 2001; Kaelin, 2002). Three different targets will be described: methionine aminopeptidase-2 (MetAP-2), estrogen receptor (ER), and androgen receptor (AR). We will provide an overview of binding assays, transfections, immunoprecipitations, and ubiquitination and degradation assays of the proteins targeted to ubiquitin ligases by Protacs.

Strategies to Assess the Efficacy of Protacs *In Vitro*

As proof of concept, we generated a Protac molecule that targets the protein MetAP-2 for ubiquitination and degradation. MetAP-2 cleaves the N-terminal methionine from nascent polypeptides and is one of the targets of angiogenesis inhibitors fumagillin and ovalicin (Griffith *et al.*, 1997; [redacted] 1998; Sin *et al.*, 1997). Ovalicin covalently binds to MetAP-2 at the His-231 active site. Inhibition of MetAP-2 is thought to block endothelial cell proliferation by causing G1 arrest (Yeh *et al.*, 2000). MetAP-2 is a stable protein that has not been demonstrated to be ubiquitinated or an endogenous substrate of $SCF^{\beta\text{-TRCP}}$. For these reasons, Met-AP2 was chosen to be the initial target to test Protacs.

The heteromeric ubiquitin ligase, $SCF^{\beta\text{-TRCP}}$ (Skp1-Cullin-Fbox-Hrt1), was selected because the F-box protein β-TRCP/E3RS was previously shown to bind to IκBα (inhibitor of NFκBα) through a minimal phosphopeptide sequence, DRHDS*GLDS*M (phosphoserines indicated by asterisks) (Ben-Neriah, 2002; Karin and Ben-Neriah, 2000). This 10-amino acid phosphopeptide was linked to ovalicin to form the Protac (Protac-1) as previously described (Sakamoto *et al.*, 2001).

MetAP-2-Protac Coupling Assay

MetAP-2 (9 μM) was incubated with increasing concentrations of Protac-1 for 45 min at room temperature (Fig. 2). Reactions were supplemented with SDS loading dye, fractionated on an SDS/10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and immunoblotted with rabbit polyclonal anti-MetAP-2 antisera (Zymed, Inc.). Detection was performed using enhanced chemiluminescence (Amersham, Inc.).

Tissue Culture

293T cells were cultured in DMEM with 10% (vol/vol) FBS (Gibco, Inc.), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM). Cells were split 1:5 before the day of transfection and transiently transfected with 40 μg of plasmid. Cells were 60% confluent in 100-mm

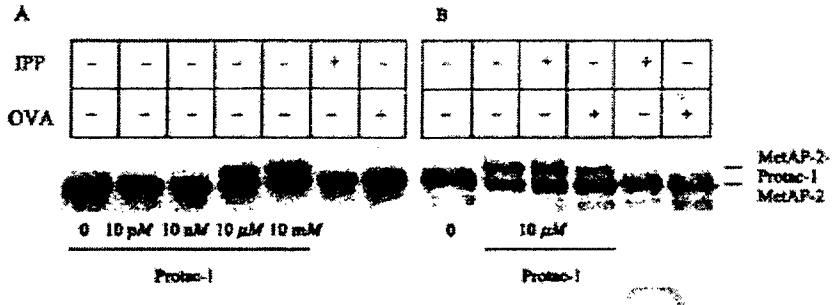


FIG. 2. MetAP-2 binds Protac specifically and in a concentration-dependent manner. (A) MetAP-2 (9 μM) was incubated with increasing concentrations of Protac-1 at room temperature for 45 min. The last two lanes depict MetAP-2 that was incubated with either free IκBα phosphopeptide (IPP; 50 μM) or free ovalicin (OVA; 50 μM), as indicated. After incubation, samples were supplemented with SDS-PAGE loading dye, fractionated by SDS-PAGE, and immunoblotted with MetAP-2 antiserum. (B) Same as (A), except MetAP-2 (9 μM) plus Protac-1 (10 μM) were supplemented with either IκBα phosphopeptide (50 μM) or ovalicin (50 μM) as indicated. Protac binding to MetAP-2 was inhibited by addition of ovalicin, but not phosphopeptide (B) (Sakamoto *et al.*, 2001).

dishes on the day of transfection. DNA (20 μg of pFLAG-CUL1 and 20 μg of pFLAG-β-TRCP) was added. Cells were transfected using calcium phosphate precipitation as previously described (Lyapina *et al.*, 1998). Cells were harvested 30 h after transfection. Five micrograms of pGL-1, a plasmid containing the cytomegalovirus (CMV) promoter linked to the green fluorescent protein (GFP) cDNA, was cotransfected into cells to determine transfection efficiency. In all experiments, greater than 80% of the cells were GFP-positive at the time of harvest, indicating high transfection efficiency.

Immunoprecipitations and Ubiquitination Assays

293T cells were lysed with 200 μl of lysis buffer (25 m*M* Tris-Cl, pH 7.5/150 m*M* NaCl/0.1% Triton X-100/5 m*M* NaF/0.05 m*M* EGTA/1 m*M* PMSF). Pellets were lysed by vortexing for 10 sec in a 4° cold room, then placed on ice for 15 min. After centrifugation at 13,000 rpm in a Microfuge for 5 min at 4°, the supernatant was added to 20 μl of FLAG M2 affinity beads (Sigma) and incubated for 2 h rotating at 4°. Beads were spun down at 13,000 rpm and washed with buffer A (25 m*M* Hepes buffer, pH 7.4/0.01% Triton X-100/150 m*M* NaCl) and one wash with buffer B (the same as buffer A but without Triton X-100). Four microliters of MetAP-2 (18 μM) stock, 4 μl of Protac-1 (100 μM), 0.5 μl of 0.1 μg/μl purified mouse E1 (Boston Biochem), 1 μl of 0.5 μg/μl human Cdc34 E2 (Boston Biochem), and 1 μl of

25 m*M* ATP were added to 20 μl (packed volume) of FLAG beads immunoprecipitated with SCF. Reactions were incubated for 1 h at 30° in a Thermomixer (Eppendorf) with constant mixing. SDS-PAGE loading buffer was added to terminate reactions, which were then evaluated by Western blot analysis as previously described (Sakamoto *et al.*, 2001) (Fig. 3). Our results demonstrated that MetAP-2 bound to Protac could be ubiquitinated *in vitro* in the presence of SCF. These methods can be generalized to other ubiquitin ligases provided that a small molecule or peptide ligand exists to enable the synthesis of a suitable Protac and expression vectors that contain tagged versions of the protein or subunits are available. Alternative tags (e.g., myc or HA) have been used, and the resin can be cross-linked with an antibody, which can then be used to immunoprecipitate the E3 ligase from mammalian cells. Both the ER and AR are members of the steroid hormone receptor superfamily whose interactions with ligand (estradiol and testosterone, respectively) have been well characterized (Fig. 4). The ER has been implicated in the progression of breast cancer (Howell *et al.*, 2003). Similarly, hormone-dependent prostate cancer cells grow in response to androgens (Debes *et al.*, 2002). Therefore, both ER and AR are logical targets for cancer therapy. To target ER for ubiquitination and degradation, a Protac

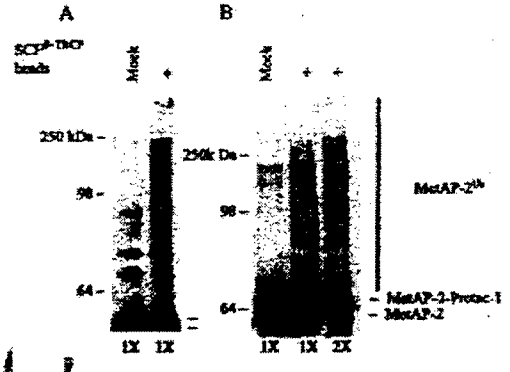


FIG. 3. Protac mediates MetAP-2 ubiquitination by SCF. (A) Ubiquitination of the 46-kDa fragment of MetAP-2. MetAP-2–Protac-1 mixture was added to either control (mock) or SCF$^{\beta\text{-TRCP}}$ beads (+) supplemented with ATP plus purified E1, E2 (Cdc34), and ubiquitin. UbcH5c (500 pg) was also tested as E2 in the reaction, which resulted in the same degree of ubiquitination as observed with Cdc34 (data not shown). Reactions were incubated for 1 h at 30°, and were evaluated by SDS-PAGE followed by Western blotting with anti-MetAP-2 antiserum. (B) Ubiquitination of full-length (67 kDa) MetAP-2. Same as (A), except that the 67-kDa preparation of MetAP-2 was used, and E1, E2, plus ubiquitin were either added at normal (1×) or twofold higher (2×) levels, as indicated (Sakamoto *et al.*, 2001).

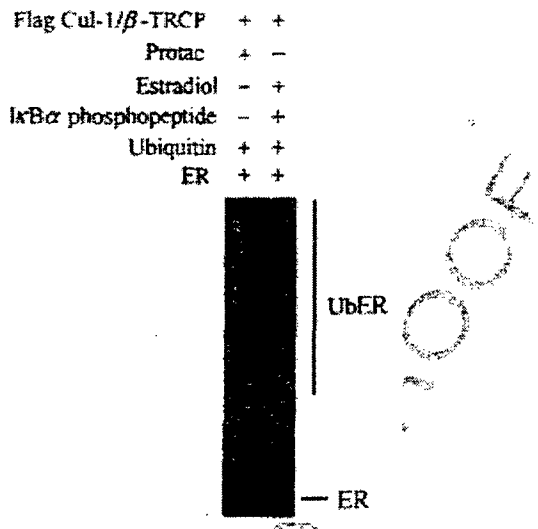


FIG. 4. Protac-2 activates ubiquitination of ER *in vitro*. Purified ER was incubated with recombinant E1, E2, ATP, ubiquitin, and immobilized $SCF^{\beta\text{-TRCP}}$ isolated from animal cells by virtue of Flag tags on cotransfected Cul1 and β-TRCP. Reactions were supplemented with the indicated concentration of Protac-2, incubated for 60 min at 30°, and monitored by SDS-PAGE followed by immunoblotting with an anti-ER antibody (Sakamoto *et al.*, 2003).

(Protac-2) was synthesized, containing the IκBα phosphopeptide linked to estradiol (the ligand for ER) (Sakamoto *et al.*, 2003).

Determination of Protein Degradation of Ubiquitinated Proteins In Vitro

The success of Protacs depends not only on efficient ubiquitination of the proposed target but also degradation of that target in cells. Several approaches can be used both *in vitro* and *in vivo* to demonstrate that the target is being destroyed. First, demonstration of degradation *in vitro* can be performed with purified 26S proteasome. For these experiments, we used purified yeast proteasomes as previously described (Verma *et al.*, 2000, 2002).

Ubiquitination assays were first performed with the immunoprecipitated E3 ligase, purified target, E1, E2, ATP, and ubiquitin with Protac. Purified yeast 26S proteasomes (40 μl of 0.5mg/ml) were added to ubiquitinated protein (e.g., ER) on beads. The reaction was supplemented with 6 μl of 1 m*M* ATP, 2 μl of 0.2 *M* magnesium acetate, and ubiquitin aldehyde (5 μ*M* final concentration). The reaction was incubated for 10 min at 30° with the occasional mixing in the Thermomixer (Eppendorf). To

verify that degradation is due to proteasomes and not other proteases, purified 26S proteasomes were preincubated for 45 min at 30° with 1 mm of 1, 10 phenanthroline (Sigma) (a metal chelator and inhibitor of the RPN11 deubiquitinating enzyme in the 26S proteasome) (Fig. 5).

Strategies to Assess the Efficacy of Protacs *In Vivo*

Clinical application of Protacs is dependent on successful ubiquitination and degradation of the protein target by endogenous ubiquitin ligases and proteasomes within cells. There are several approaches to test the efficacy of Protacs using cell extracts or application directly to cells. Depending on the polarity of the Protac, efficiency of internalization in cells is variable. If Protacs are hydrophilic, such as the case with the Protac-1 that contains the IκBα phosphopeptide, extracts or microinjections are possible approaches. For cell-permeable Protacs, it is be possible to directly bath apply Protacs to cells.

Degradation Experiments with Xenopus *Extracts*

Extracts from unfertilized *Xenopus laevis* eggs were prepared on the day of the experiment as previously described (Murray, 1991). MetAP-2 (4 μl of 9 μM) was incubated with Protac-1 (50 μM) at room temperature for 45

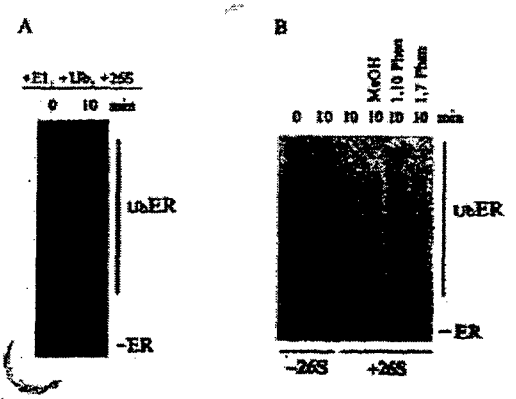


FIG. 5. Ubiquitinated ER is degraded by the 26S proteasome. (A) Ubiquitination reactions performed as described in the legend to Fig. 5A were supplemented with purified yeast 26S proteasomes. Within 10 min, complete degradation of ER was observed. (B) Purified 26S proteasome preparations were preincubated in 1,10 phenanthroline (1 mM) or 1,7 phenanthroline (1 mM) before addition. The metal chelator 1,10 phenanthroline inhibits the Rpn11-associated deubiquitinating activity that is required for substrate degradation by the proteasome. Degradation of ER was partially inhibited by addition of 1,10 phenanthroline, but not the inactive derivative 1,7 phenanthroline (Sakamoto *et al.*, 2003).

min. The MetAP-2–Protac-1 mixture was added to 10 μl of extract in addition to excess ovalicin (10 μM final concentration). The excess of ovalicin was added to saturate any free MetAP-2 in the reaction. Additional components in the reaction included constitutively active IKK (IKK-EE; 0.4 μg) and okadaic acid (10 μM final concentration) to maintain phosphorylation of the IκBα peptide moiety of Protac. To test for specificity of proteasomal degradation, various proteasome inhibitors were used, including N-acetyl-leu-leu-norleucinal (LLnL, 50 μM final) or epoxomicin (10 μM final). Protease inhibitors chymotrypsin, pepstatin, and leupeptin cocktail (15 μg/ml final concentration) were also added to the extracts. Reactions were incubated for time points up to 30 min at room temperature and terminated by adding 50 μl of SDS loading buffer. Samples were then evaluated by Western blot analysis using MetAP-2 antiserum (Fig. 6).

Microinjection as a Method to Study Effects of Protacs on Ubiquitination and Degradation of Target Proteins

Protacs that contain a phosphopeptide do not enter cells efficiently. Various protein transduction domains, lipid-based transfection reagents, and electroporation or other transient transfection methods can be tested. However, to facilitate Protacs entering cells, microinjections were performed. For these experiments, Protac-3 (IκBα) phosphopeptide-testosterone was synthesized to target the AR (Sakamoto *et al.*, 2003). As a readout of protein degradation, 293 cells stably expressing AR-GFP were selected using G418 (600 μg/ml). Before microinjections, cells were approximately 60% confluent in 6-cm dishes.

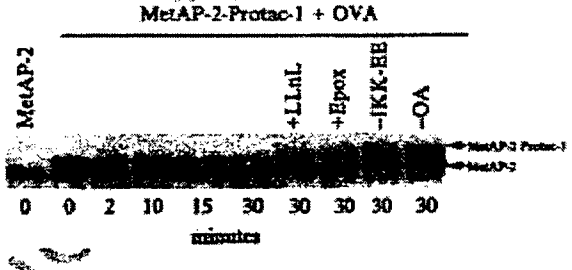
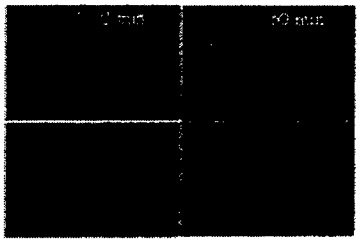


FIG. 6. MetAP-2-Protac but not free MetAP-2 is degraded in *Xenopus* extracts. The MetAP-2–Protac-1 mixture or MetAP-2 alone was added to *Xenopus* egg extract fortified with ovalicin (OVA; 100 μM), IKK-EE (0.4 μg), and okadaic acid (10 μM). Where indicated, reactions were either deprived of IKK-EE or okadaic acid (OA) or were further supplemented with 50 μM LLnL or 10 μM epoxomicin (Epox). Reactions were incubated for the indicated time points at room temperature, terminated by adding SDS-PAGE loading dye, and evaluated by SDS-PAGE followed by Western blotting with anti-MetAP-2 antiserum (Sakamoto *et al.*, 2001).

Protac-3 diluted in a KCl solution (10 μM final) with rhodamine dextran (molecular mass 10,000 Da; 50 μg/ml) was injected into cells through a microcapillary needle using a pressurized injection system (Picospritzer II; General Valve Corporation). Coinjection with rhodamine dextran is critical to ensure that decrease in AR-GFP is not due to leakage of protein from cells after microinjection. The injected volume was 0.2 pl, representing 5–10% of the cell volume. GFP and rhodamine fluorescence can be visualized with a fluorescent microscope (Zeiss) and photographs taken with an attached camera (Nikon). Within 1 h after microinjection, disappearance of AR-GFP is visible (Fig. 7). Cells should remain rhodamine

A



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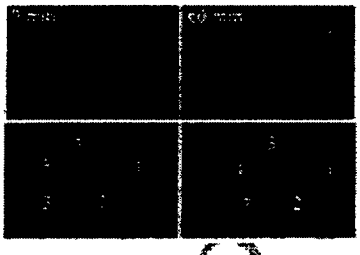

Degree of AR-GFP Disappearance	Percent (out of >200 cells)
1. NONE	4
2. MINIMAL	16
3. PARTIAL	29
4. COMPLETE	51

FIG. 7. Microinjection of Protac leads to AR-GFP degradation in cells. Protac-3 (10 μM in the microinjection needle) was introduced using a Picospritzer II pressurized microinjector into $293^{AR\text{-}GFP}$ cells in a solution containing KCl (200 μM) and rhodamine dextran (50 μg/ml). Approximately 10% of total cell volume was injected. (A) Protac-3 induces AR-GFP disappearance within 60 min. The top panels show cell morphology under light microscopy overlaid with images of cells injected with Protac as indicated by rhodamine fluorescence (pink color). The bottom panels show images of GFP fluorescence. By 1 h, GFP signal disappeared in almost all microinjected cells. To quantify these results, we injected more than 200 cells and classified the degree of GFP disappearance as being either none (1), minimal (2), partial (3), or complete (4). Examples from each category and the tabulated results are shown in (B). These results were reproducible in three independent experiments performed on separate days with 30–50 cells injected per day (Sakamoto *et al.*, 2003). (See color insert.)

positive provided that injection has not caused lysis of cells or leakage of AR-GFP from cells. Greater than 200 cells per experiment (in three separate experiments) provide data demonstrating that Protacs induces degradation of the target. AR-GFP disappearance can then be quantitated by categorizing the intensity of GRP signal as indicative of complete disappearance, partial disappearance, minimal disappearance, or no disappearance. To verify that the disappearance of AR-GFP from cells is proteasome dependent, cells were pretreated with proteasome inhibitor epoxomicin (10 μM final) for 5 h before microinjections or were coinjected with epoxomicin (10 μM).

Methods to Test a Cell-Permeable Protac

Reagents capable of redirecting the substrate specificity of the ubiquitin-proteasome pathway in protein degradation would be useful experimental tools for modulating cellular phenotype and potentially acting as drugs to eliminate disease-promoting proteins. To use Protacs to remove a gene product at the posttranslational level, a cell-permeable reagent would be necessary. A HIF1α-DHT Protac was developed for this purpose. Given the lack of small molecule E3 ligase ligands, the seven amino acid sequence ALAPYIP from hypoxia-inducible factor 1α (HIF1α) was chosen for the E3 recognition domain of Protac-4 (Schneekloth *et al.*, 2004). This sequence has been demonstrated to be the minimum recognition domain for the von Hippel-Lindau tumor suppressor protein (VHL) (Hon *et al.*,

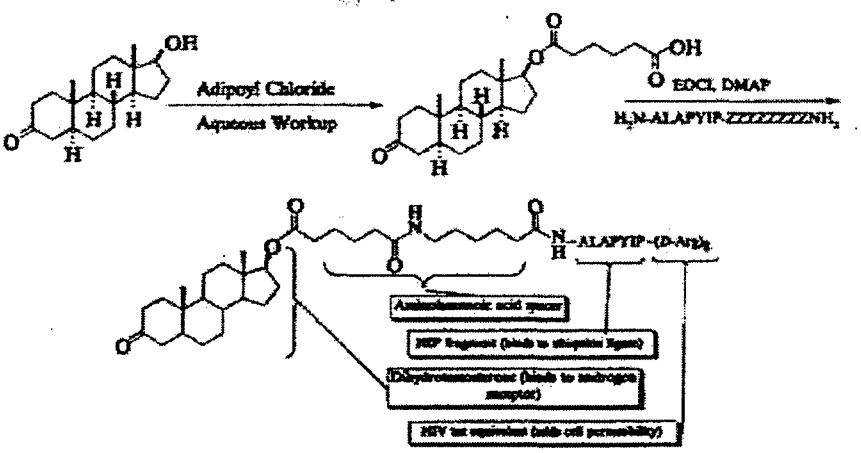

FIG. 8. Chemical Structure of HIF-DHT Protac (Schneekloth *et al.*, 2004).

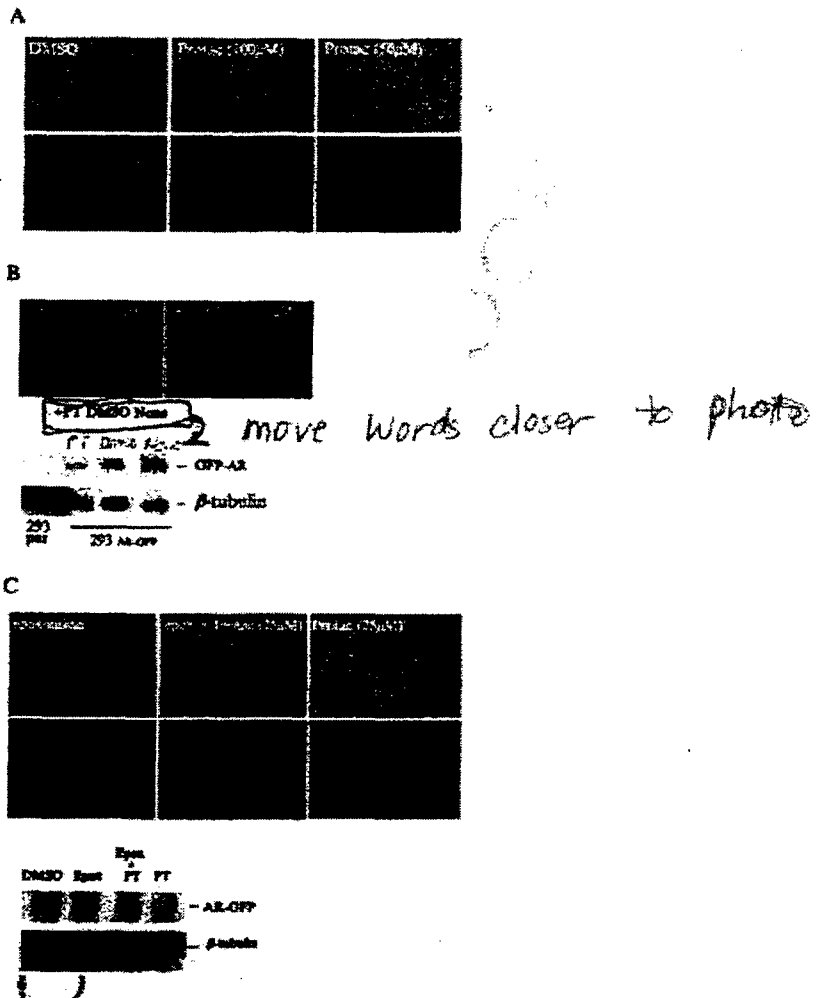

FIG. 9. HIF-DHT Protac mediates AR-GFP degradation in a proteasome-dependent manner. $293^{AR\text{-}GFP}$ cells (0.5×10^6 cells/ml) were plated at 50% confluence in a volume of 200 μl of media in a 96-well dish. (A and B) Protac induces AR-GFP disappearance within 60 min. Protac in a 100-, 50-, or 25-μM concentration or DMSO control in a volume of 0.6 μl was added. Cells were visualized under light (top) or fluorescent (bottom) microscopy 1 h after treatment. Photographs were taken with a SC35 type 12, 35-mm camera attached to an Olympus fluorescent inverted microscope. (B) AR-GFP protein is decreased in cells treated with Protac. Lysates were prepared from parental cells (293 par) or AR-GFP expressing cells treated with Protac (+PT), DMSO, or no treatment (None) for 60 min. Western blot analysis was performed with rabbit polyclonal anti-AR antisera (1:1000; UBI) or β-tubulin (1:200;

2002; Kaelin, 2002). VHL is part of the VBC-Cul2 E3 ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of HIF1α at the (Epstein *et al.*, 2001) central proline in the ALAPYIP sequence. This modification results in recognition and polyubiquitination by VHL. HIF1α is constitutively ubiquitinated and degraded under normoxic conditions (Kaelin, 2002). In addition, a poly-D-arginine tag derived from HIV tat was added to the carboxy terminus of the peptide sequence to confer cell permeability and prevent nonspecific proteolysis (Kirschberg *et al.*, 2003; Wender *et al.*, 2000) (Fig. 8). This Protac should then enter the cell, be recognized and hydroxylated by a prolyl hydroxylase, and subsequently be bound by both the VHL E3 ligase and the target, AR.

The 293 cells stably expressing AR-GFP were used to study the effects of HIF1α-DHT Protac on AR degradation. For these experiments, greater than 95% of cells expressed AR-GFP. On the day before experiments, cells were plated in 96-well plates with 200 μl of media at 60% confluence. Protac was dissolved in DMSO and was added to cells at concentrations ranging between 10 μM–100 μM. The presence or absence of GFP expression after Protac treatment was determined by fluorescent microscopy. A time course was performed, but for HIF1α-DHT Protac, the effects were observed within 2 h. To assess proteasome-dependent degradation, cells were pretreated with epoxomicin (10 μM final concentration) for 4 h before adding Protac. Western blot analysis was performed to determine levels of AR-GFP (Fig. 9).

To measure the protein levels of AR-GFP after Protac treatment, the cells were harvested, washed with PBS once, then pelleted at 1500 rpm. Cells were lysed with boiling SDS loading buffer (30 μl), then boiled for 5 min. Lysates were subjected to 8% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane. Western blot analysis was performed with antiandrogen receptor (1:1000) and anti-beta tubulin (1:200) antisera. Detection was determined using chemiluminescence.

Santa Cruz). (C) Epoxomicin inhibits Protac-induced degradation of AR-GFP. Cells were plated at a density of 0.3×10^6 cells/ml and treated with 10 μM epoxomicin (Calbiochem) or DMSO for 4 h before adding Protac (25 μM) for 60 min. (D) Western blot analysis was performed with cells in 96-well dishes treated with Protac (25 μM), DMSO (left), epoxomicin (10 μM), epoxomicin (10 μM) + Protac (50 or 25 μM), or Protac alone (50 or 25 μM) (Schneekloth *et al.*, 2004). (See color insert.)

Acknowledgments

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